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(21) International Application Number: PCT/US99/02390 (22) International Filing Date: 3 February 1999 (03.02.99) (30) Priority Data: 09/020,598 4 February 1998 (04.02.98) US (71) Applicants: GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, CA 94080-4990 (US). UNIVERSITY OF IOWA RESEARCH FOUNDATION [US/US]; Technology Innovation Center, Room 214 TIC, 100 Oakdale Campus, Iowa City, IA 52242-5000 (US). (72) Inventors: SLIWKOWSKI, Mark; 42 Oak Creek Lane, Iowa City, IA 94070 (US). KERN, Jeffrey, A.; 4344 Maier Avenue, Iowa City, IA 52240 (US). (74) Agents: SCHWARTZ, Timothy, R. et al.; Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080-4990 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: USE OF HEREGULIN AS AN EPITHELIAL CELL GROWTH FACTOR (57) Abstract Ligands which bind to the HER2, HER3 and/or HER4 receptors are useful as normal epithelial cell growth factors.		

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USE OF HEREGULIN AS AN EPITHELIAL CELL GROWTH FACTOR

BACKGROUND OF THE INVENTION**Field of the Invention**

This invention relates to the use of HER2, HER3 and/or HER4 ligands, in particular heregulin polypeptides, as epithelial cell growth factors.

Description of Background and Related Art

The HER (ErbB) family belongs to the subclass I receptor tyrosine kinase superfamily and consists of three distinct receptors, HER2, HER3, and HER4. A ligand for this ErbB family is the protein heregulin (HRG), a multidomain containing protein with at least 15 distinct isoforms.

Transduction of signals that regulate cell growth and differentiation is regulated in part by phosphorylation of various cellular proteins. Protein tyrosine kinases are enzymes that catalyze this process. Receptor protein tyrosine kinases are believed to direct cellular growth via ligand-stimulated tyrosine phosphorylation of intracellular substrates. Growth factor receptor protein tyrosine kinases of the class I subfamily include the 170 kDa epidermal growth factor receptor (EGFR) encoded by the *erbB1* gene. *erbB1* has been causally implicated in human malignancy. In particular, increased expression of this gene has been observed in more aggressive carcinomas of the breast, bladder, lung and stomach.

The second member of the class I subfamily, p185^{neu} was originally identified as the product of the transforming gene from neuroblastomas of chemically treated rats. The *neu* gene (also called *erbB2* and HER2) encodes a 185 kDa receptor protein tyrosine kinase. Amplification and/or overexpression of the human HER2 gene correlates with a poor prognosis in breast and ovarian cancers (Slamon *et al.*, *Science* 235:177-182 (1987); and Slamon *et al.*, *Science* 244:707-712 (1989)). Overexpression of HER2 has been correlated with other carcinomas including carcinomas of the stomach, endometrium, salivary gland, lung, kidney, colon and bladder. Accordingly, Slamon *et al.* in US Pat No. 4,968,603 describe and claim various diagnostic assays for determining HER2 gene amplification or expression in tumor cells. Slamon *et al.* discovered that the presence of multiple gene copies of HER2 oncogene in tumor cells indicates that the disease is more likely to spread beyond the primary tumor site, and that the disease may therefore require more aggressive treatment than might otherwise be indicated by other diagnostic factors. Slamon *et al.* conclude that the HER2 gene amplification test, together with the determination of lymph node status, provides greatly improved prognostic utility.

A further related gene, called *erbB3* or HER3, has also been described. See US Pat. No. 5,183,884; Kraus *et al.*, *Proc. Natl. Acad. Sci. USA* 86:9193-9197 (1989); EP Pat Appln No 444,961A1; and Kraus *et al.*, *Proc. Natl. Acad. Sci. USA* 90:2900-2904 (1993). Kraus *et al.* (1989) discovered that markedly elevated levels of *erbB3* mRNA were present in certain human mammary tumor cell lines indicating that *erbB3*, like *erbB1* and *erbB2*, may play a role in human malignancies. Also, Kraus *et al.* (1993) showed that EGF-dependent activation of the ErbB3 catalytic domain of a chimeric EGFR/ErbB3 receptor resulted in a proliferative response in transfected NIH-3T3 cells. This is now believed to be the result of endogenous ErbB1 or ErbB2 in NIH-3T3. Furthermore, these researchers demonstrated that some human mammary tumor cell lines display a significant elevation of steady-state ErbB3 tyrosine phosphorylation further indicating that this receptor may play a role in human malignancies. The role of *erbB3* in cancer has been explored by others. It has been found to be overexpressed in breast (Lemoine *et al.*, *Br. J. Cancer* 66:1116-1121 (1992)), gastrointestinal (Poller *et al.*, *J. Pathol.* 168:275-280 (1992), Rajkumar *et al.*, *J. Pathol.* 170:271-278 (1993), and Sanidas *et al.*, *Int. J. Cancer* 54:935-940 (1993)), and pancreatic cancers (Lemoine *et al.*, *J. Pathol.* 168:269-273 (1992), and Friess *et al.*, *Clinical Cancer Research* 1:1413-1420 (1995)).

The class I subfamily of growth factor receptor protein tyrosine kinases has been further extended to

include the HER4/Erb4 receptor. See EP Pat Appln No 599,274; Plowman *et al.*, *Proc. Natl. Acad. Sci. USA* 90:1746-1750 (1993); and Plowman *et al.*, *Nature* 366:473-475 (1993). Plowman *et al.* found that increased HER4 expression closely correlated with certain carcinomas of epithelial origin, including breast adenocarcinomas. Diagnostic methods for detection of human neoplastic conditions (especially breast cancers) which evaluate HER4 expression are described in EP Pat Appln No. 599,274.

The quest for the activator of the HER2 oncogene has lead to the discovery of a family of heregulin polypeptides. These proteins appear to result from alternate splicing of a single gene which was mapped to the short arm of human chromosome 8 by Orr-Urtreger *et al.*, *Proc. Natl. Acad. Sci. USA* 90:1867-1871(1993). See also Lee and Wood, *Genomics*, 16:790-791 (1993).

Holmes *et al.* isolated and cloned a family of polypeptide activators for the HER2 receptor which they called heregulin- α (HRG- α), heregulin- β 1 (HRG- β 1), heregulin- β 2 (HRG- β 2), heregulin- β 2-like (HRG- β 2-like), and heregulin- β 3 (HRG- β 3). See Holmes *et al.*, *Science* 256:1205-1210 (1992); WO 92/20798; and U.S. Patent 5,367,060. The 45 kDa polypeptide, HRG- α , was purified from the conditioned medium of the MDA-MB-231 human breast cancer cell line. These researchers demonstrated the ability of the purified heregulin polypeptides to activate tyrosine phosphorylation of the HER2 receptor in MCF7 breast tumor cells. Furthermore, the mitogenic activity of the heregulin polypeptides on SK-BR-3 cells (which express high levels of the HER2 receptor) was illustrated. Like other growth factors which belong to the EGF family, soluble HRG polypeptides appear to be derived from a membrane bound precursor (called pro-HRG) which is proteolytically processed to release the 45kDa soluble form. These pro-HRGs lack a N-terminal signal peptide.

While heregulins are substantially identical in the first 213 amino acid residues, they are classified into two major types, α and β , based on two variant EGF-like domains which differ in their C-terminal portions. Nevertheless, these EGF-like domains are identical in the spacing of six cysteine residues contained therein. Based on an amino acid sequence comparison, Holmes *et al.* found that between the first and sixth cysteines in the EGF-like domain, HRGs were 45% similar to heparin-binding EGF-like growth factor (HB-EGF), 35% identical to amphiregulin (AR), 32% identical to TGF- α , and 27% identical to EGF.

The 44 kDa *neu* differentiation factor (NDF), which is the rat equivalent of human HRG, was first described by Peles *et al.*, *Cell*, 69:205-216 (1992); and Wen *et al.*, *Cell*, 69:559-572 (1992). Like the HRG polypeptides, NDF has an immunoglobulin (Ig) homology domain followed by an EGF-like domain and lacks a N-terminal signal peptide. Subsequently, Wen *et al.*, *Mol. Cell. Biol.*, 14(3):1909-1919 (1994) carried out "exhaustive cloning" to extend the family of NDFs. This work revealed six distinct fibroblastic pro-NDFs. Adopting the nomenclature of Holmes *et al.*, the NDFs are classified as either α or β polypeptides based on the sequences of the EGF-like domains. Isoforms 1 to 4 are characterized on the basis of the variable justamembrane stretch (between the EGF-like domain and transmembrane domain). Also, isoforms a, b and c are described which have variable length cytoplasmic domains. These researchers conclude that different NDF isoforms are generated by alternative splicing and perform distinct tissue-specific functions. See also EP 505 148; WO 93/22424; and WO 94/28133 concerning NDF.

Falls *et al.*, *Cell*, 72:801-815 (1993) describe another member of the heregulin family which they call acetylcholine receptor inducing activity (ARIA) polypeptide. The chicken-derived ARIA polypeptide stimulates synthesis of muscle acetylcholine receptors. See also WO 94/08007. ARIA is a β -type heregulin and lacks the entire spacer region rich in glycosylation sites between the Ig-like domain and EGF-like domain of HRG α , and HRG β 1- β 3.

Marchionni *et al.*, *Nature*, 362:312-318 (1993) identified several bovine-derived proteins which they call glial growth factors (GGFs). These GGFs share the Ig-like domain and EGF-like domain with the other heregulin proteins described above, but also have an amino-terminal kringle domain. GGFs generally

do not have the complete glycosylated spacer region between the Ig-like domain and EGF-like domain. Only one of the GGFs, GGFII, possessed a N-terminal signal peptide. See also WO 92/18627; WO 94/00140; WO 94/04560; WO 94/26298; and WO 95/32724 which refer to GGFs and uses thereof.

Ho *et al.* in *J. Biol. Chem.* 270(4):14523-14532 (1995) describe another member of the heregulin family called sensory and motor neuron-derived factor (SMDF). This protein has an EGF-like domain characteristic of all other heregulin polypeptides but a distinct N-terminal domain. The major structural difference between SMDF and the other heregulin polypeptides is the lack in SMDF of the Ig-like domain and the "glyco" spacer characteristic of all the other heregulin polypeptides. Another feature of SMDF is the presence of two stretches of hydrophobic amino acids near the N-terminus.

While the heregulin polypeptides were first identified based on their ability to activate the HER2 receptor (see Holmes *et al.*, *supra*), it was discovered that certain ovarian cells expressing *neu* and *neu*-transfected fibroblasts did not bind or crosslink to NDF, nor did they respond to NDF to undergo tyrosine phosphorylation (Peles *et al.*, *EMBO J.* 12:961-971 (1993)). This indicated another cellular component was necessary for conferring full heregulin responsiveness. Carraway *et al.* subsequently demonstrated that ¹²⁵I-

rHRGβ₁₇₇₋₂₄₄ bound to NIH-3T3 fibroblasts stably transfected with bovine *erbB3* but not to non-transfected parental cells. Accordingly, they conclude that ErbB3 is a receptor for HRG and mediates phosphorylation of intrinsic tyrosine residues as well as phosphorylation of ErbB2 receptor in cells which express both receptors. Carraway *et al.*, *J. Biol. Chem.* 269(19):14303-14306 (1994). Sliwkowski *et al.*, *J. Biol. Chem.* 269(20):14661-14665 (1994) found that cells transfected with HER3 alone show low affinities for heregulin, whereas cells transfected with both HER2 and HER3 show higher affinities.

This observation correlates with the "receptor cross-talking" described previously by Kokai *et al.*, *Cell* 58:287-292 (1989); Stern *et al.*, *EMBO J.* 7:995-1001 (1988); and King *et al.*, 4:13-18 (1989). These researchers found that binding of EGF to the EGFR resulted in activation of the EGFR kinase domain and cross-phosphorylation of p185^{HER2}. This is believed to be a result of ligand-induced receptor heterodimerization and the concomitant cross-phosphorylation of the receptors within the heterodimer (Wada *et al.*, *Cell* 61:1339-1347 (1990)).

Plowman and his colleagues have similarly studied p185^{HER4}/p185^{HER2} activation. They expressed p185^{HER2} alone, p185^{HER4} alone, or the two receptors together in human T lymphocytes and demonstrated that heregulin is capable of stimulating tyrosine phosphorylation of p185^{HER4}, but could only stimulate p185^{HER2} phosphorylation in cells expressing both receptors. Plowman *et al.*, *Nature* 336:473-475 (1993).

The biological role of heregulin has been investigated by several groups. For example, Falls *et al.*, (discussed above) found that ARIA plays a role in myotube differentiation, namely affecting the synthesis and concentration of neurotransmitter receptors in the postsynaptic muscle cells of motor neurons. Corfas and Fischbach demonstrated that ARIA also increases the number of sodium channels in chick muscle. Corfas and Fischbach, *J. Neuroscience*, 13(5): 2118-2125 (1993). It has also been shown that GGFII is mitogenic for subconfluent quiescent human myoblasts and that differentiation of clonal human myoblasts in the continuous presence of GGFII results in greater numbers of myotubes after six days of differentiation (Sklar *et al.*, *J. Cell Biochem.*, Abst. W462, 18D, 540 (1994)). See also WO 94/26298 published November 24, 1994.

Holmes *et al.*, *supra*, found that HRG exerted a mitogenic effect on mammary cell lines (such as SK-BR-3 and MCF-7). The mitogenic activity of GGFs on Schwann cells has also been reported. See, e.g., Brockes *et al.*, *J. Biol. Chem.* 255(18):8374-8377 (1980); Lemke and Brockes, *J. Neurosci.* 4:75-83 (1984); Lemke and Brockes, *J. Neurosci.* 4:75-83 (1984); Brockes *et al.*, *Ann. Neurol.* 20(3):317-322 (1986); Brockes, J., *Methods in Enzym.*, 147: 217-225 (1987) and Marchionni *et al.*, *supra*. Schwann cells constitute

important glial cells which provide myelin sheathing around the axons of neurons, thereby forming individual nerve fibers. Thus, it is apparent that Schwann cells play an important role in the development, function and regeneration of peripheral nerves. The implications of this from a therapeutic standpoint have been addressed by Levi *et al.*, *J. Neuroscience* 14(3):1309-1319 (1994). Levi *et al.* discuss the potential for construction of a cellular prosthesis comprising human Schwann cells which could be transplanted into areas of damaged spinal cord. Methods for culturing Schwann cells *ex vivo* have been described. See WO 94/00140 and Li *et al.*, *J. Neuroscience* 16(6):2012-2019 (1996).

Pinkas-Kramarski *et al.* found that NDF seems to be expressed in neurons and glial cells in embryonic and adult rat brain and primary cultures of rat brain cells, and suggested that it may act as a survival and maturation factor for astrocytes (Pinkas-Kramarski *et al.*, *PNAS, USA* 91:9387-9391 (1994)). Meyer and Birchmeier, *PNAS, USA* 91:1064-1068 (1994) analyzed expression of heregulin during mouse embryogenesis and in the perinatal animal using in situ hybridization and RNase protection experiments. See also Meyer *et al.*, *Development* 124(18):3575-3586 (1997). These authors conclude that, based on expression of this molecule, heregulin plays a role *in vivo* as a mesenchymal and neuronal factor. Similarly, Danilenko *et al.*, Abstract 3101, *FASEB* 8(4-5):A535 (1994); Danilenko *et al.*, *Journal of Clinical Investigation* 95(2):842-851 (1995), found that the interaction of NDF and the HER2 receptor is important in directing epidermal migration and differentiation during wound repair.

Ram *et al.*, *Journal of Cellular Physiology* 163:589-596 (1995) evaluated the mitogenic activity of NDF on the immortalized human mammary epithelial cell line MCF-10A. Danilenko *et al.*, *J. Clin. Invest.* 95:842-851 (1995) investigated whether NDF would influence epidermal migration in an *in vivo* model of excisional deep partial-thickness wound repair. It is reported that there were no statistically significant differences in proliferating basal and superbasal keratinocytes in control wounds vs. wounds treated with rhNDF- α_2 . Marikovsky *et al.*, *Oncogene* 10:1403-1411 (1995), studied the proliferative responses of an aneuploid BALB/MK continuous keratinocyte cell line and evaluated the effects of α - and β -isoforms of NDF on epidermal keratinocytes.

The relationship between the structure and function of new proteins can be investigated using any of a variety of available mutational analysis techniques. Examples of such techniques include alanine scanning mutagenesis and phagemid display. Alanine scanning can be used to identify active residues (i.e., residues that have a significant effect on protein function) in a protein or protein domain. For example, Cunningham and Wells used alanine scanning to identify residues in human growth hormone that were important for binding its receptor. Cunningham and Wells, *Science* 244:1081-1085 (1989). In alanine scanning, a gene encoding the protein or domain to be scanned is inserted into an expression vector, and mutagenesis is carried out to generate a series of vectors that encode proteins or domains in which sequential residues are converted to alanine. The encoded proteins or domain are expressed from these vectors, and the activities of the alanine-substituted variants are then tested to identify those with altered activity. An alteration in activity indicates that the residue at the alanine-substituted position is an active residue.

Phagemid display was developed to allow the screening of a large number of variant polypeptides for a particular binding activity. Smith and Parmley demonstrated that foreign peptides can be "displayed" efficiently on the surface of filamentous phage by inserting short gene fragments into gene III of the fd phage. Smith, *Science* 228:1315-1317 (1985); Parmley and Smith, *Gene* 73:305-318 (1985). The gene III coat protein is present in about five copies at one end of the phage particle. The modified phage were termed "fusion phage" because they displayed the foreign peptides fused to the gene III coat protein. As each fusion phage particle displayed approximately five copies of the fusion protein, this mode of phage display was termed "polyvalent display."

Scott *et al.* and Cwirla *et al.* showed that fusion phage libraries could be screened by sequential affinity selections known as "panning." Scott *et al.*, *Science* 249:386-390 (1990); Cwirla *et al.*, *PNAS USA*

87:6378-6382 (1990). However, early efforts to select high affinity fusion phage failed, presumably due to the polyvalence of the phage particles. This problem was solved with the development of a "monovalent" phage display system in which the fusion protein is expressed at a low level from a phagemid and a helper phage provides a large excess of wild-type coat protein. Bass *et al.*, *Proteins* 8:309-314 (1990); Lowman *et al.*, *Biochem.* 30:10832-10838 (1991). Monovalent phage display can be used to generate and screen a large number of variant polypeptides to isolate those that bind with high affinity to a target of interest.

Approximately 50,000 infants are born in the United States every year with birth weights, less than 1.5 kg. About two thirds of these very low birth weight infants have evidence of pulmonary immaturity manifested as respiratory distress shortly after birth. The majority of these infants require mechanical ventilation. Respiratory distress syndrome, caused by insufficient pulmonary surfactant production, as well as structural immaturity of the lung, is responsible for respiratory difficulties observed in these prematurely born neonates. Well developed alveoli are necessary to provide efficient oxygen transfer from the air-liquid interface of the lung to the systemic circulation. Surfactant proteins are critical in reducing the alveolar surface tension at low lung volumes and preventing alveolar collapse.

A need continues to exist for a method of treatment for respiratory distress syndrome and other diseases associated with immature lung development and low lung surfactant production.

SUMMARY OF THE INVENTION

In general an object of the invention is to provide a method of inducing epithelial cell growth and development for the purpose of promoting repair and healing of tissue damage or injury.

Accordingly, one object of this invention is to provide a method of treating respiratory distress syndrome in patients, primarily human patients, in need of such treatment. A further object is to provide a method of inducing lung epithelial cell growth and development. A further object is to provide a method of increasing lung surfactant protein A production in the lung of persons with impaired oxygen transfer in the lung alveoli. This invention is useful in treating infants/neonates with respiratory distress as well as youth and adults with poor lung function due to lung injury or damage.

In one aspect of this invention, it has now been discovered that these objects and the broader objective of treating conditions associated with epithelial cell damage and injury are achieved by administering to a patient in need of such treatment an effective amount of a heregulin ligand, preferably a polypeptide or fragment thereof. These heregulin (HRG) polypeptides, include HRG- α , HRG- β 1, HRG- β 2, HRG- β 3 and other HRG polypeptides which cross-react with antibodies directed against these family members and/or which are substantially homologous as defined below and includes HRG variants such as N-terminal and C-terminal fragments thereof. A preferred HRG is the ligand disclosed in Fig. 1A - 1D and further designated HRG- α . Other preferred HRGs are the ligands disclosed in Figure 2A - 2E, and designated HRG- β 1; disclosed in Figure 3A - 3E designated HRG- β 2; and disclosed in Figure 4A - 4C designated HRG- β 3.

In another aspect, the invention provides a method in which HRG agonist antibodies are administered to achieve the objects of the invention. In this embodiment, HER2/HER3 or fragments thereof (which also may be synthesized by *in vitro* methods) are fused (by recombinant expression or an *in vitro* peptidyl bond) to an immunogenic polypeptide and this fusion polypeptide, in turn, is used to raise antibodies against a HER2/HER3 epitope. Agonist antibodies are recovered from the serum of immunized animals. Alternatively, monoclonal antibodies are prepared from *in vitro* cells or *in vivo* immunized animals in conventional fashion. If desired, the agonist antibodies may be obtained by phage display selection from a phage library of antibodies or antibody fragments. Preferred antibodies identified by routine screening will bind to the receptor, but will not substantially cross-react with any other known ligands such as EGF, and will activate the HER receptors HER2, HER3 and/or HER4. In addition, antibodies may be selected that are

capable of binding specifically to individual family members of HRG family, e.g. HRG- α , HRG- β 1, HRG- β 2, HRG- β 3, and which are agonists thereof.

In general, the invention is a method of regenerating and/or repairing epithelial cell injury by stimulating growth and proliferation of epithelial cells, in particular ductal and ciliated epithelial cells. The epithelial cells may be injured by many types of insults, for example, injury due to surgical incision or resection, chemical or smoke inhalation or aspiration, chemical or biochemical ulceration, cell damage due to viral or bacterial infection, etc. The epithelial cells which may be affected by the method of the invention include any epithelial cell which expresses HER2, HER3 and/or HER4; suitable cells are located, for example, in the lung, gastric mucosa, endometrium, oviducts, mammary glands, pancreas, salivary glands, etc. The method of the invention stimulates growth and proliferation of the epithelial cells, repairing and re-establishing the cellular barriers of organs and allowing the affected tissues to develop normal physiological functions more quickly. For example, lung epithelial cells are damaged by inhalation of smoke resulting in emphysema. Treatment of the lung cells by the method of the invention regenerates the barrier layer of lung epithelial cells, improves oxygenation and speeds the development of a barrier to infection. Similarly, cell damage due to aspiration of gastric acid can be treated by the method of the invention to facilitate regeneration of epithelial cells.

Accordingly, one embodiment of the invention is a method of inducing lung epithelial cell growth and development by contacting a lung epithelial cell which expresses HER2, HER3 and/or HER4 receptors with an effective amount of a HER2, HER3 and/or HER4 activating ligand.

Another embodiment is a method of increasing lung surfactant protein A in a patient by administering to a patient in need thereof an effective amount of a HER2, HER3 and/or HER4 activating ligand.

A further embodiment is a method of treating respiratory distress by administering to a patient in need thereof an effective amount of a HER2, HER3 and/or HER4 activating ligand.

A further embodiment is a method of treating emphysema by administering to a patient in need thereof an effective amount of a HER2, HER3 and/or HER4 activating ligand.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A - 1D show the deduced amino acid sequence (SEQ ID NO:1) for the cDNA sequence (SEQ ID NO:2) contained in a clone obtained according to U.S. 5,367,060. The initiating methionine (Met) of HRG- α is at position 45.

Figure 2A - 2E show the deduced amino acid sequence (SEQ ID NO:3) and cDNA sequence (SEQ ID NO:4) of a potential coding sequence of a clone obtained according to U.S. 5,367,060 for HRG- β 1. The initiating Met is at M31.

Figure 3A - 3E show the deduced amino acid sequence (SEQ ID NO:5) and cDNA sequence (SEQ ID NO:6) of a nucleotide sequence of a clone obtained according to U.S. 5,367,060 for HRG- β 2.

Figure 4A - 4C show the deduced amino acid sequence (SEQ ID NO:7) and cDNA sequence (SEQ ID NO:8) of a nucleotide sequence of a clone obtained according to U.S. 5,367,060 for HRG- β 3.

Figure 5A - 5D show the deduced amino acid sequence (SEQ ID NO:9) and cDNA sequence (SEQ ID NO:10) of a nucleotide sequence of a clone obtained according to U.S. 5,367,060 for HRG- β 2-like protein.

Figure 6A - 6C show a comparison of the amino acid homologies of several known heregulins α , β 1, β 2, β 2-like and β 3 in descending order and illustrates the amino acid insertions, deletions, and substitutions that characterize these forms of HRG (SEQ ID NOS: 1, 3, 5, 9, and 7).

Figure 7A - 7C show the deduced amino acid sequence (SEQ ID NO:11) and cDNA sequence (SEQ

ID NO:12) of γ -HRG obtained as described in U.S. Serial No. 08/891,845. The hydrophobic region is underlined. The EGF-like domain is shaded, cysteine residues in the EGF-like domain are circled. N-linked glycosylation sites are marked above the nucleic acid sequence with a ().

Figure 8 shows the cDNA sequence (SEQ ID NO:13) and amino acid sequence (SEQ ID NO:14) of SMDF obtained as described in U.S. Serial No. 08/339,517. An EGF-like domain and the apolar and uncharged domains (*i.e.* "apolar I" consisting of residues from about 48-62 and "apolar II" consisting of residues from about 76-100) are underlined. Cysteines in the EGF-like domain and in the "cysteine knot" in the unique N-terminal domain ("NTD-cys knot") are boxed. The stop codon is denoted by the letter "O".

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

HRG ligands, in particular polypeptides and agonist antibodies thereof, have affinity for and stimulate the HER2, HER3 and/or HER4 receptors or combinations thereof in autophosphorylation. Included within the definition of HRG ligands, in addition to HRG- α , HRG- β 1, HRG- β 2, HRG- β 3 and HRG- β 2-like, are other polypeptides binding to the HER2, HER3 and/or HER4 receptor, which bear substantial amino acid sequence homology to HRG- α or HRG- β 1. Such additional polypeptides fall within the definition of HRG as a family of polypeptide ligands that bind to the HER2, HER3 and/or HER4 receptors.

Heregulin polypeptides bind with varying affinities to the HER2, HER3 and/or HER4 receptors. Generally, the HER3 and HER4 receptors are bound with high affinity. It is also known that heterodimerization of HER2 with HER3 and of HER2 with HER4 occurs with subsequent receptor cross-phosphorylation as described above. In the present invention, epithelial cell growth and/or proliferation is induced when a heregulin protein interacts and binds with an individual receptor molecule or a receptor dimer such that receptor phosphorylation is induced. Binding and activation of HER2, HER3, HER4 or combinations thereof, therefore, is meant to include activation of any form of the receptor necessary for receptor activation and biologic function including monomeric receptor and dimeric receptor forms. Dimeric receptor forms may be referred to below, for example, as HER2/HER3, HER2/HER4, and HER3/HER4.

I. DEFINITIONS

In general, the following words or phrases have the indicated definition when used in the description, examples, and claims.

"Heregulin" (HRG) ligand is defined herein to be any isolated ligand, preferably a polypeptide sequence which possesses a biological property of a naturally occurring HRG polypeptide. Ligands within the scope of this invention include the NDF, ARIA and GGF growth factor heregulin proteins identified above as well as the SMDF and HRG polypeptides discussed in detail herein. These isolated NDF, ARIA and GGF heregulin polypeptides are well known in the art. HRG includes the polypeptides shown in Figs. 1A-1D, 2A-2E, 3A-3E, 4A-4C, 5A-5D, 6A-6C, 7A-7C and 8 and mammalian analogues thereof. Included are HRG variants such as the γ -HRG described in WO 98/02541, published 22 January 1998; the variants described in WO 98/35036, published 13 August 1998; and the SMDF variants described in US Patent NO. 5,770,567 granted 23 June 1998 (WO 96/15244). These applications are incorporated herein in their entirety. These variants can be prepared by the methods described below, optionally together with alanine scanning and phage display techniques known in the art. Cunningham and Wells, Science 244:1081-85 (1989); Bass et al., Proteins 8:309-14 (1990); Lowman et al., Biochem. 30:10832-38 (1991).

The term a "normal" epithelial cell means an epithelial cell which is not transformed, *i.e.*, is non-cancerous and/or non-immortalized. Further, the normal epithelial cell is preferably not aneuploid. Aneuploidy exists when the nucleus of a cell does not contain an exact multiple of the haploid number of chromosomes, one or more chromosomes being present in greater or lesser number than the rest. Typical properties of transformed cells which fall outside the scope of this invention include the ability to form

tumors when implanted into immune-deprived mice (nude mice), the ability to grow in suspension or in semi-solid media such as agar, a loss of contact inhibition allowing piling up of cells into colonies or foci, a loss of dependence on growth factors or serum, cell death if cells are inhibited from growing, and disorganization of actin filaments. Specifically included within the invention are normal epithelial cells which will not form tumors in mice, grow attached to plastic or glass (are anchorage dependent), exhibit contact inhibition, require serum-containing hormones and growth factors, remain viable if growth is arrested by lack of serum, and contain well-organized actin filaments. Although the normal epithelial cells are preferably not cultured cells, also suitable for the invention are non-transformed, non-immortalized epithelial cells isolated from mammalian tissue. These isolated cells may be cultured for several generations (up to about 10 or even 50 generations) in the presence of a heregulin in order to induce growth and/or proliferation of the isolated epithelial cell sample, that is, to expand the sample. The expanded sample can then be reintroduced into the mammal for the purpose of repopulating the epithelial cell tissue (re-epithelialization). This is particularly useful for repairing tissue injury or damage.

An "epithelial" cell is a cell located in a cellular, avascular layer covering the free surface (cutaneous, mucous or serous) of an organ or lining a tube or cavity of an animal body. Lung epithelial cells include bronchial epithelial cells, Type II cells and Clara cells. The term "epithelial cell" as used herein is consistent with the art recognized definition of epithelial cells in epithelium. See, for example, the definition in Taber's Encyclopedic Medical Dictionary, Edition 12, (1973) F.A. Davis Company, publisher.

"Biological property" for the purposes herein means an *in vivo* biologic or antigenic function or activity that is directly or indirectly performed by an HRG sequence (whether in its native or denatured conformation), or by any subsequence thereof. Biologic functions include receptor binding, any enzyme activity or enzyme modulatory activity, any carrier binding activity, any hormonal activity, any activity in promoting or inhibiting adhesion of cells to extracellular matrix or cell surface molecules, or any structural role. However, biologic functions do not include antigenic functions, i.e. possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against a naturally occurring HRG polypeptide.

"Biologically active" HRG is defined herein as a polypeptide sharing a biologic function of an HRG sequence which may (but need not) in addition possess an antigenic function. A principal known effect or function of HRG is as a ligand polypeptide having a qualitative biological activity of binding to HER2, HER3 and/or HER4 resulting in the activation of the receptor tyrosine kinase (an "activating ligand"). One test for activating ligands is the HRG tyrosine autophosphorylation assay described below. Included within the scope of HRG as that term is used herein are HRG having translated mature amino acid sequences of the complete human HRG as set forth herein; deglycosylated or unglycosylated derivatives of HRG, amino acid sequence variants of HRG sequence, and derivatives of HRG, which are capable of exhibiting a biological property in common with HRG. While native HRG is a membrane-bound polypeptide, soluble forms, such as those forms lacking a functional transmembrane domain, are also included within this definition. In particular, included are polypeptide fragments of HRG sequence which have an N-terminus at any residue from about S216 to about A227, and its C-terminus at any residue from about K268 to about R286, and the homologous sequences shown in Fig. 6A-C, hereinafter referred to collectively for all HRGs as the growth factor domain (GFD).

"Antigenically active" HRG is defined as a polypeptide that possesses an antigenic function of an HRG and which may (but need not) in addition possess a biologic function.

In preferred embodiments, antigenically active HRG is a polypeptide that binds with an affinity of at least about 10^{-9} l/mole to an antibody raised against a naturally occurring HRG sequence. Ordinarily the polypeptide binds with an affinity of at least about 10^{-8} l/mole. Most preferably, the antigenically active HRG is a polypeptide that binds to an antibody raised against one of HRGs in its native conformation. HRG

in its native conformation generally is HRG as found in nature which has not been denatured by chaotropic agents, heat or other treatment that substantially modifies the three dimensional structure of HRG as determined, for example, by migration on nonreducing, nondenaturing sizing gels. Antibody used in this determination may be rabbit polyclonal antibody raised by formulating native HRG from a non-rabbit species in Freund's complete adjuvant, subcutaneously injecting the formulation, and boosting the immune response by intraperitoneal injection of the formulation until the titer of anti-HRG antibody plateaus.

Ordinarily, biologically or antigenically active HRG will have an amino acid sequence having at least 75% amino acid sequence identity with a given HRG sequence, more preferably at least 80%, even more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to an HRG sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with HRG residues in the HRG of Fig. 6A-6C, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal or internal extensions, deletions, or insertions into HRG sequence shall be construed as affecting homology.

Thus, the biologically active and antigenically active HRG polypeptides that are the subject of this invention include each entire HRG sequence; fragments thereof having a consecutive sequence of at least 5, 10, 15, 20, 25, 30 or 40 amino acid residues from HRG sequence; amino acid sequence variants of HRG sequence wherein an amino acid residue has been inserted N- or C-terminal to, or within, HRG sequence or its fragment as defined above; amino acid sequence variants of HRG sequence or its fragment as defined above has been substituted by another residue. HRG polypeptides include those containing predetermined mutations by, e.g., site-directed or PCR mutagenesis, and other animal species of HRG polypeptides such as rabbit, rat, porcine, non-human primate, equine, murine, and ovine HRG and alleles or other naturally occurring variants of the foregoing and human sequences; derivatives of HRG or its fragments as defined above wherein HRG or its fragments have been covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid (for example a detectable moiety such as an enzyme or radioisotope); glycosylation variants of HRG (insertion of a glycosylation site or deletion of any glycosylation site by deletion, insertion or substitution of appropriate amino acid); and soluble forms of HRG, such as HRG-GFD or those that lack a functional transmembrane domain.

"Isolated" means a ligand, such as HRG, which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for HRG, and may include proteins, hormones, and other substances. In preferred embodiments, HRG will be purified (1) to greater than 95% by weight of protein as determined by the Lowry method or other validated protein determination method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of the best commercially available amino acid sequenator marketed on the filing date hereof, or (3) to homogeneity by SDS-PAGE using Coomassie blue or, preferably, silver stain. Isolated HRG includes HRG in situ within recombinant cells since at least one component of HRG natural environment will not be present. Isolated HRG includes HRG from one species in a recombinant cell culture of another species since HRG in such circumstances will be devoid of source polypeptides. Ordinarily, however, isolated HRG will be prepared by at least one purification step.

In accordance with this invention, HRG nucleic acid is RNA or DNA containing greater than ten bases that encodes a biologically or antigenically active HRG, is complementary to nucleic acid sequence encoding such HRG, or hybridizes to nucleic acid sequence encoding such HRG and remains stably bound to it under stringent conditions.

Preferably, HRG nucleic acid encodes a polypeptide sharing at least 75% sequence identity, more preferably at least 80%, still more preferably at least 85%, even more preferably at 90%, and most preferably

95%, with an HRG sequence. Preferably, the HRG nucleic acid that hybridizes contains at least 20, more preferably at least about 40, and most preferably at least about 90 bases.

Isolated HRG nucleic acid includes a nucleic acid that is identified and separated from at least one containment nucleic acid with which it is ordinarily associated in the natural source of HRG nucleic acid.

5 Isolated HRG nucleic acid thus is present in other than in the form or setting in which it is found in nature. However, isolated HRG encoding nucleic acid includes HRG nucleic acid in ordinarily HRG-expressing cells where the nucleic acid is in a chromosomal location different from that of natural cells or is otherwise flanked by a different DNA sequence than that found in nature. Nucleic acid encoding HRG may be used in specific hybridization assays, particularly those portions of HRG encoding sequence that do not hybridize
10 with other known DNA sequences. "Stringent conditions" are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% NaDodSO₄ at 50° C; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42° C; or (3) employ 50%
15 formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42° C, with washes at 42° C in 0.2 x SSC and 0.1% SDS.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for
20 prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA
25 for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However enhancers do not have to be
30 contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

An "exogenous" element is defined herein to mean nucleic acid sequence that is foreign to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is ordinarily not found.

35 As used herein, the expressions "cell", "cell line", and "cell culture" are used interchangeably, and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the
40 originally transformed cell are included. It will be clear from the context where distinct designations are intended.

"Plasmids" are designated by a lower case "p" preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed from such available plasmids in accord with published procedures. In addition,
45 other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

"Restriction enzyme digestion" of DNA refers to catalytic cleavage of the DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction endonucleases, and the sites for which each is specific is called a restriction site. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements as established by the enzyme suppliers are used. Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters representing the microorganism from which each restriction enzyme originally was obtained, and then a number designating the particular enzyme. In general, about 1 mg of plasmid or DNA fragment is used with about 1-2 units of enzyme in about 20 ml of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation of about 1 hour at 37°C is ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein or polypeptide is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme may be followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction cleaved ends of a DNA fragment from "circularizing" or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional as described in sections 1.56-1.61 of Sambrook *et al.*, (*Molecular Cloning: A Laboratory Manual* New York: Cold Spring Harbor Laboratory Press, 1989).

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see Lawn *et al.*, *Nucleic Acids Res.* 9:6103-6114 (1981), and Goeddel *et al.*, *Nucleic Acids Res.* 8:4057 (1980).

"Northern analysis" is a method used to identify RNA sequences that hybridize to a known probe such as an oligonucleotide, DNA fragment, cDNA or fragment thereof, or RNA fragment. The probe is labeled with a radioisotope such as ³²P, or by biotinylation, or with an enzyme. The RNA to be analyzed is usually electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe, using standard techniques well known in the art such as those described in sections 7.39-7.52 of Sambrook *et al.*, *supra*.

"Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments. To ligate the DNA fragments together, the ends of the DNA fragments must be compatible with each other. In some cases, the ends will be directly compatible after endonuclease digestion. However, it may be necessary to first convert the staggered ends commonly produced after endonuclease digestion to blunt ends to make them compatible for ligation. To blunt the ends, the DNA is treated in a suitable buffer for at least 15 minutes at 15°C with about 10 units of the Klenow fragment of DNA polymerase I or T4 DNA polymerase in the presence of the four deoxyribonucleotide triphosphates. The DNA is then purified by phenol-chloroform extraction and ethanol precipitation. The DNA fragments that are to be ligated together are put in solution in about equimolar amounts. The solution will also contain ATP, ligase buffer, and a ligase such as T4 DNA ligase at about 10 units per 0.5 mg of DNA. If the DNA is to be ligated into a vector, the vector is first linearized by digestion with the appropriate restriction endonuclease(s). The linearized fragment is then treated with bacterial alkaline phosphatase, or calf intestinal phosphatase to prevent self-ligation during the ligation step.

"Preparation" of DNA from cells means isolating the plasmid DNA from a culture of the host cells. Commonly used methods for DNA preparation are the large and small-scale plasmid preparations described

in sections 1.25-1.33 of Sambrook *et al.*, *supra*. After preparation of the DNA, it can be purified by methods well known in the art such as that described in section 1.40 of Sambrook *et al.*, *supra*.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid phase techniques such as described in EP 266,032, published 4 May 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler *et al.*, *Nucl. Acids Res.* 14:5399-5407, 1986. They are then purified on polyacrylamide gels.

The technique of "polymerase chain reaction," or "PCR," as used herein generally refers to a procedure wherein minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Pat. No. 4,683,195, issued 28 July 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. See generally Mullis *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* 51: 263 (1987); Erlich, ed., *PCR Technology*, (Stockton Press, NY, 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample, comprising the use of a known nucleic acid (DNA or RNA) as a primer, and utilizes a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid or to amplify or generate a specific piece of nucleic acid which is complementary to a particular nucleic acid.

The "HRG tyrosine autophosphorylation assay" to detect the presence or bioactivity of HRG ligands can be used to monitor the purification of a ligand for the HER2 and HER3 receptors. This assay is based on the assumption that a specific ligand for the receptor will stimulate autophosphorylation of the receptor, in analogy with EGF and its stimulation of EGF receptor autophosphorylation. See Sadich *et al.*, *Anal. Biochem.* 235:207-214 (1996). MDA-MB-453 cells or MCF7 cells which contain high levels of p185^{HER2} receptors but negligible levels of human EGF receptors, were obtained from the American Type Culture Collection, Rockville, Md. (ATCC No HTB-131) and maintained in tissue culture with 10% fetal calf serum in DMEM/Hams F12 (1:1) media. For assay, the cells were trypsinized and plated at 150,000 cells/well in 24 well dishes (Costar). After incubation with serum containing media overnight, the cells were placed in serum free media for 2-18 hours before assay. Test samples of 100 uL aliquots were added to each well. The cells were incubated for 5-30 minutes (typically 30 min) at 37°C and the media removed. The cells in each well were treated with 100 uL SDS gel denaturing buffer (SEPROSOL, Enpotech, Inc.) and the plates heated at 100°C for 5 minutes to dissolve the cells and denature the proteins. Aliquots from each well were electrophoresed on 5-20% gradient SDS gels (NOVEX, Encinitas, CA) according to the manufacturer's directions. After the dye front reached the bottom of the gel, the electrophoresis was terminated and a sheet of PVDF membrane (PROBLOTT, ABI) was placed on the gel and the proteins transferred from the gel to the membrane in a blotting chamber (BioRad) at 200 mAmps for 30-60 min. After blotting, the membranes were incubated with TRIS buffered saline containing 0.1% TWEEN 20 detergent buffer with 5% BSA for 2-18 hrs to block nonspecific binding, and then treated with a mouse anti-phosphotyrosine antibody (Upstate Biological Inc., N.Y.). Subsequently, the membrane blots were treated with goat anti-mouse antibody conjugated to alkaline phosphatase. The gels were developed using the PROTOBLOT System from Promega. After drying the membranes, the density of the bands corresponding to p185^{HER2} in each sample lane was quantitated with a Hewlett Packard SCANJET Plus Scanner attached to a Macintosh computer. The number of receptors per cell in the MDA-MB-453 cells is such that under these experimental conditions the p185^{HER2} receptor protein is the major protein which is labeled.

"Protein microsequencing" was accomplished based upon the following procedures. Proteins from the final HPLC step were either sequenced directly by automated Edman degradation with a model 470A Applied Biosystems gas phase sequencer equipped with a 120A PTH amino acid analyzer or sequenced after digestion with various chemicals or enzymes. PTH amino acids were integrated using the CHROMPERFECT data system (Justice Innovations, Palo Alto, CA). Sequence interpretation was performed on a VAX 11/785 Digital Equipment Corporation computer as described (Henzel *et al.*, *J. Chromatography* 404:41-52 (1987)). In some cases, aliquots of the HPLC fractions were electrophoresed on 5-20% SDS polyacrylamide gels, electrotransferred to a PVDF membrane (PROBLOTT, ABI, Foster City, CA) and stained with Coomassie Brilliant Blue (Matsudaira, P., *J. Biol. Chem.* 262:10035-10038, 1987). The specific protein was excised from the blot for N terminal sequencing. To determine internal protein sequences, HPLC fractions were dried under vacuum (SPEEDVAC), resuspended in appropriate buffers, and digested with cyanogen bromide, the lysine-specific enzyme Lys-C (Wako Chemicals, Richmond, VA) or Asp-N (Boehringer Mannheim, Indianapolis, Ind.). After digestion, the resultant peptides were sequenced as a mixture or were resolved by HPLC on a C4 column developed with a propanol gradient in 0.1% TFA before sequencing as described above.

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H - V_L dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteine(s) from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ , respectively. The subunit structures and three-dimensional

configurations of different classes of immunoglobulins are well known.

The term "antibody" herein is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

5 "Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable domain thereof. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

10 The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a
15 substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.*, *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567). The
20 "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, *Nature* 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences
25 in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

30 "Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances,
35 framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The
40 humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al.*, *Nature* 321:522-525 (1986); Reichmann *et al.*, *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein
45 these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for

antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain ($V_H - V_L$). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments ($V_H - C_H1 - V_H - C_H1$) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

II. USE AND PREPARATION OF HRG SEQUENCES

H. PREPARATION OF HRG SEQUENCES, INCLUDING VARIANTS

The system to be employed in preparing HRG sequence will depend upon the particular HRG sequence selected. If the sequence is sufficiently small HRG may be prepared by *in vitro* polypeptide synthetic methods. Most commonly, however, HRG will be prepared in recombinant cell culture using the host-vector systems described below. Suitable HRG includes any biologically active and antigenetically active HRG.

In general, mammalian host cells will be employed, and such hosts may or may not contain post-translational systems for processing HRG preprosequences in the normal fashion. If the host cells contain such systems then it will be possible to recover natural subdomain fragments such as HRG-GFD from the cultures. If not, then the proper processing can be accomplished by transforming the hosts with the required enzyme(s) or by supplying them in an *in vitro* method. However, it is not necessary to transform cells with the complete prepro or structural genes for a selected HRG when it is desired to only produce fragments of HRG sequences such as an HRG-GFD. For example, a start codon is ligated to the 5' end of DNA encoding an HRG-GFD, this DNA is used to transform host cells and the product expressed directly as the Met N-terminal form (if desired, the extraneous Met may be removed *in vitro* or by endogenous N-terminal demethionylases). Alternatively, HRG-GFD is expressed as a fusion with a signal sequence recognized by the host cell, which will process and secrete the mature HRG-GFD as is further described below. Amino acid sequence variants of native HRG-GFD sequences are produced in the same way.

HRG sequences located between the first N-terminal mature residue and the first N-terminal residue of HRG-GFD sequence, termed HRG-NTD, may function at least in part as an unconventional signal sequence or as a normally circulating carrier/precursor for HRG-GFD having unique biological activity.

HRG-NTD is produced in the same fashion as the full length molecule but from expression of DNA in which a stop codon is located at the C-terminus of HRG-NTD. In addition, HRG variants are expressed from DNA encoding protein in which both the GFD and NTD domains are in their proper orientation but which contain an amino acid insertion, deletion or substitution at the GFD-NTD cleavage site (located within the sequence VKC) which inhibits or prevents proteolytic cleavage of the NTD-GFD joining site *in vivo*, and wherein a stop codon is positioned at the 3' end of the GFD- encoding sequence. In an example of this group of variants (termed HRG-NTDXGFD), (1) the lysine residue found in the NTD-GFD joining sequence VKC is deleted or (preferably) substituted by another residue other than arginyl such as histidyl, alanyl, threonyl or seryl and (2) a stop codon is introduced in the sequence RCT or RCQ in place of cysteinyl, or threonyl (for HRG- α) or glutaminyl (for HRG- β).

A preferred HRG- α ligand with binding affinity to p185HER2 comprises amino acids 226-265 of figure 1A-D. This HRG- α ligand further may comprise up to an additional 1-20 amino acids preceding amino acid 226 and 1-20 amino acids following amino acid 265. A preferred HRG- β ligand with binding affinity to p185HER2 comprises amino acids 226-265 of figure 2A-E. This HRG- β ligand may comprise up to an additional 1-20 amino acids preceding amino acid 226 and 1-20 amino acids following amino acid 265.

As noted above, other HRG sequences to be prepared in accordance with this invention are those of the GFD. These are synthesized *in vitro* or are produced in recombinant cell culture. These are produced most inexpensively in yeast or *E.coli* by secretion under the control of a HRG-heterologous signal as described *infra*, although preparation in mammalian cells is also contemplated using a mammalian protein signal such as that of tPA, UK or a secreted viral protein. The GFD can be the sequence of a native HRG or may be a variant thereof as described below. GFD sequences include those in which one or more residues from a member of the EGF family are substituted into or onto the GFD sequence.

An additional HRG is one which contains the GFD and the sequence between the C-terminus of GFD and the N-terminus of the transmembrane domain (the later being termed the C-terminal cleavage domain or CTC). In this variant (HRG-GFD-CTC) the DNA start codon is present at the 5' end of HRG-heterologous signal sequence or adjacent the 5' end of the GFD-encoding region, and a stop codon is found in place of one of the first about 1 to 3 extra-cellular domain (ECD) residues or first about 1-2 transmembrane region residues. In addition, in some HRG-GFD-CTC variants the codons are modified in the GFD-CTC proteolysis site by substitution, insertion or deletion. The GFD-CTC proteolysis site is the domain that contains the GFD C-terminal residue and about 5 residues N- and 5 residues C-terminal from this residue. It is known that Met-227 terminal and Val-229 terminal HRG- α -GFD are biologically active. The C-terminus for HRG- α -GFD may be Met-227, Lys-228, Val-229, Gln-230, Asn-231 or Gln-232, and for HRG- β -GFD may be Met-226, Ala-227, Ser-228, Phe-229, Trp-230, or Lys231/Ser231. The native C-terminus is determined readily by C- terminal sequencing, although it is not critical that HRG-GFD have the native terminus so long as the GFD sequence possesses the desired activity. In some embodiments of HRG-GFD-CTC variants, the amino acid change(s) in the CTC are screened for their ability to resist proteolysis *in vitro* and inhibit the protease responsible for generation of HRG-GFD.

HRG-ECD variants are made by providing a stop codon at the same location as for HRG-GFD-CTC variants. HRG-ECD may comprise any one or more of the variants described above in connection with its subfragments, e.g. the GFD-CTC variants containing CTC-proteolysis site modifications.

If it is desired to prepare the longer HRG polypeptides and the 5' or 3' ends of the given HRG are not described herein, it may be necessary to prepare nucleic acids in which the missing domains are supplied by homologous regions from more complete HRG nucleic acids. Alternatively, the missing domains can be obtained by probing libraries using the DNAs disclosed in the Figures or fragments thereof.

A. Isolation of DNA Encoding Heregulin

The DNA encoding HRG may be obtained from any cDNA library prepared from tissue believed to possess HRG mRNA and to express it at a detectable level. HRG- α gene thus may be obtained from a genomic library. Similar procedures may be used for the isolation of other HRG, such as HRG- β 1, HRG- β 2, or HRG- β 3 encoding genes.

Libraries are screened with probes designed to identify the gene of interest or the protein encoded by it. For cDNA expression libraries, suitable probes include monoclonal or polyclonal antibodies that recognize and specifically bind to HRG- α ; oligonucleotides of about 20-80 bases in length that encode known or suspected portions of HRG- α cDNA from the same or different species; and/or complementary or homologous cDNAs or fragments thereof that encode the same or a similar gene. Appropriate probes for

screening genomic DNA libraries include, but are not limited to, oligonucleotides; cDNAs or fragments thereof that encode the same or a similar gene; and/or homologous genomic DNAs or fragments thereof. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in chapters 10-12 of Sambrook *et al.*, *supra*.

5 An alternative means to isolate the gene encoding HRG- α is to use polymerase chain reaction (PCR) methodology as described in section 14 of Sambrook *et al.*, *supra*. This method requires the use of oligonucleotide probes that will hybridize to HRG- α . Strategies for selection of oligonucleotides are described below.

10 Another alternative method for obtaining the gene of interest is to chemically synthesize it using one of the methods described in Engels *et al.* (*Agnew. Chem. Int. Ed. Engl.*, 28: 716-734, 1989). These methods include triester, phosphite, phosphoramidite and H-Phosphonate methods, PCR and other autoprimer methods, and oligonucleotide syntheses on solid supports. These methods may be used if the entire nucleic acid sequence of the gene is known, or the sequence of the nucleic acid complementary to the coding strand is available, or alternatively, if the target amino acid sequence is known, one may infer potential nucleic acid sequences using known and preferred coding residues for each amino acid residue.

15 A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various tissues, preferably human breast, colon, salivary gland, placental, fetal, brain, and carcinoma cell lines. Other biological sources of DNA encoding an heregulin-like ligand include other mammals and birds. Among the preferred mammals are members of the following orders: bovine, ovine, equine, murine, and rodentia.

20 The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The actual nucleotide sequence(s) may, for example, be based on conserved or highly homologous nucleotide sequences or regions of HRG- α . The oligonucleotides may be degenerate at one or more positions. The use of degenerate oligonucleotides may be of particular importance where a library is screened from a species in which preferential codon usage in that species is not known. The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use ^{32}P -labeled ATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

25 30 Of particular interest is HRG- α nucleic acid that encodes a full-length polypeptide. In some preferred embodiments, the nucleic acid sequence includes the native HRG- α signal sequence. Nucleic acid having all the protein coding sequence is obtained by screening selected cDNA or genomic libraries, and, if necessary, using conventional primer extension procedures as described in section 7.79 of Sambrook *et al.*, *supra*, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

35 HRG- α encoding DNA of Figures 1A-1D may be used to isolate DNA encoding the analogous ligand from other animal species via hybridization employing the methods discussed above. The preferred animals are mammals, particularly bovine, ovine, equine, feline, canine and rodentia, and more specifically rats, mice and rabbits.

40 **B. Amino Acid Sequence Variants of Heregulin**

Amino acid sequence variants of HRG are prepared by introducing appropriate nucleotide changes into HRG DNA, or by *in vitro* synthesis of the desired HRG polypeptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence shown for human HRG sequences. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics. Excluded from the

scope of this invention are HRG variants or polypeptide sequences that are not novel and unobvious over the prior art. The amino acid changes also may alter post-translational processes of HRG- α , such as changing the number or position of glycosylation sites, altering the membrane anchoring characteristics, altering the intra-cellular location of HRG by inserting, deleting, or otherwise affecting the leader sequence of the native HRG, or modifying its susceptibility to proteolytic cleavage.

The HRG sequence may be proteolytically processed to create a number of HRG fragments. HRG-GFD sequences of HRG- α all contain the amino acid sequence between HRG- α cysteine 226 and cysteine 265. The amino terminus of HRG- α fragment may result from the cleavage of any peptide bond between alanine 1 and cysteine 226, preferably adjacent to an arginine, lysine, valine, or methionine, and most preferably between methionine 45 and serine 46. The carboxy terminus of HRG- α fragment may result from the cleavage of any peptide bond between cysteine 265, preferably adjacent to an arginine, lysine, valine, or methionine, and most preferably between lysine 272 and valine 273, between lysine 278 and alanine 279, or between lysine 285 and arginine 286. The resulting HRG- α ligands resulting from such proteolytic processing are the preferred ligands.

HRG- β -GFD's are analogous to those discussed above for HRG- α -GFD's. Each HRG- β -GFD contains the polypeptide segment from cysteine 212 to cysteine 251 of figure 2A-E. The amino terminus of HRG- β 1 fragment may result from the cleavage of any peptide bond between alanine 1 and cysteine 212, preferably adjacent to an arginine, lysine, valine, or methionine, and most preferably between methionine 31 and serine 32. The carboxy terminus of HRG- β 1 fragment may result from the cleavage of any peptide bond between cysteine 251 of Fig. 2A-2E, preferably adjacent to an arginine, lysine, valine, or methionine, and most preferably between valine 255 and methionine 256, between lysine 261 and histidine 262, between lysine 276 and alanine 277, or between lysine 301 and threonine 302. The resulting HRG- β 1 ligands resulting from such proteolytic processing are among the preferred ligands. Similarly, processing to produce preferred fragment ligands of HRG- β 2 based upon the Fig. 3A-3E and HRG- β 3 based upon Fig. 4A-4C may be accomplished by cleaving HRG sequences of Figs. 3A-3E and 4A-4C preferably adjacent to an arginine, lysine, valine or methionine.

In designing amino acid sequence variants of HRG, the location of the mutation site and the nature of the mutation will depend on HRG characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of other receptor ligands adjacent to the located site.

A useful method for identification of certain residues or regions of HRG polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (*Science*, 244: 1081-1085, 1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimize the performance of a mutation at a given site, ala scanning or random mutagenesis may be conducted at the target codon or region and the expressed HRG variants are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. These are variants from HRG sequence, and may

represent naturally occurring alleles (which will not require manipulation of HRG DNA) or predetermined mutant forms made by mutating the DNA, either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon HRG characteristic to be modified. Obviously, such variations that, for example, convert HRG into a known receptor ligand, are not included within the scope of this invention, nor are any other HRG variants or polypeptide sequences that are not novel and unobvious over the prior art.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically about 1 to 5 are contiguous. Deletions may be introduced into regions of low homology with other EGF family precursors to modify the activity of HRG. Deletions from HRG in areas of substantial homology with other EGF family sequences will be more likely to modify the biological activity of HRG more significantly. The number of consecutive deletions will be selected so as to preserve the tertiary structure of HRG in the affected domain, e.g., cysteine crosslinking, beta-pleated sheet or alpha helix.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within HRG sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, and most preferably 1 to 3. Examples of terminal insertions include HRG with an N-terminal methionyl residue (an artifact of the direct expression of HRG in bacterial recombinant cell culture), and fusion of a heterologous N-terminal signal sequence to the N-terminus of HRG to facilitate the secretion of mature HRG from recombinant host cells. Such signal sequences generally will be obtained from, and thus be homologous to, the intended host cell species. Suitable sequences include STII, tPA or lpp for *E. coli*, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells.

Other insertional variants of HRG include the fusion to the N- or C-terminus of HRG of an immunogenic polypeptide, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli trp* locus, or yeast protein, bovine serum albumin, and chemotactic polypeptides. C-terminal fusions of HRG-ECD with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, as described in WO 89/02922, published 6 April 1989 are contemplated.

Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in HRG molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s) of HRG, and sites where the amino acids found in HRG ligands from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity. A likely sub-domain of HRG-GFD having biological activity as a growth factor is the C-terminal segment, in particular within the sequence about from glycine 218 to valine 226 (HRG- α), and glycine 218 to lysine 228/serine 228 (HRG- β) based upon analogy to the EGF sub-sequence found to have EGF activity.

Other sites of interest are those in which particular residues of HRG-like ligands obtained from various species are identical. These positions may be important for the biological activity of HRG. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of "preferred substitutions". If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

TABLE I

	Original Residue	Exemplary Substitutions	Preferred Substitutions
	Ala (A)	val; leu; ile	val
5	Arg (R)	lys; gln; asn	lys
	Asn (N)	gln; his; lys; arg	gln
	Asp (D)	glu	glu
	Cys (C)	ser	ser
	Gln (Q)	asn	asn
10	Glu (E)	asp	asp
	Gly (G)	pro	pro
	His (H)	asn; gln; lys; arg	arg
	Ile (I)	leu; val; met; ala; phe; norleucine	leu
15	Leu (L)	norleucine; ile; val; met; ala; phe	ile
	Lys (K)	arg; gln; asn	arg
	Met (M)	leu; phe; ile	leu
	Phe (F)	leu; val; ile; ala	leu
20	Pro (P)	gly	gly
	Ser (S)	thr	thr
	Thr (T)	ser	ser
	Trp (W)	tyr	tyr
	Tyr (Y)	trp; phe; thr; ser	phe
25	Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in function or immunological identity of HRG are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

- 1) hydrophobic: norleucine, met, ala, val, leu, ile;
- 2) neutral hydrophilic: cys, ser, thr;
- 3) acidic: asp, glu;
- 4) basic: asn, gln, his, lys, arg;
- 5) residues that influence chain orientation: gly, pro; and
- 6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another. Such substituted residues may be introduced into regions of HRG that are homologous with other receptor ligands, or, more preferably, into the non-homologous regions of the molecule.

In one embodiment of the invention, it is desirable to inactivate one or more protease cleavage sites that are present in the molecule. These sites are identified by inspection of the encoded amino acid sequence. Where protease cleavage sites are identified, they are rendered inactive to proteolytic cleavage by substituting the targeted residue with another residue, preferably a basic residue such as glutamine or a hydrophilic residue such as serine; by deleting the residue; or by inserting a prolyl residue immediately after the residue.

In another embodiment, any methionyl residue other than the starting methionyl residue of the signal sequence, or any residue located within about three residues N- or C-terminal to each such methionyl residue, is substituted by another residue (preferably in accord with Table I) or deleted. Alternatively, about 1-3 residues are inserted adjacent to such sites.

5 Any cysteine residues not involved in maintaining the proper conformation of HRG also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

Sites particularly suited for substitutions, deletions or insertions, or use as fragments, include, numbered from the N-terminus of HRG- α of Figure 1A - 1D:

- 10 1) potential glycosaminoglycan addition sites at the serine-glycine dipeptides at 42-43, 64-65, 151-152;
- 2) potential asparagine-linked glycosylation at positions 164, 170, 208 and 437, sites (NDS) 164-166, (NIT) 170-172, (NTS) 208-210, and NTS (609-611);
- 3) potential O-glycosylation in a cluster of serine and threonine at 209-218;
- 15 4) cysteines at 226, 234, 240, 254, 256 and 265;
- 5) transmembrane domain at 287-309;
- 6) loop 1 delineated by cysteines 226 and 240;
- 7) loop 2 delineated by cysteines 234 and 254;
- 8) loop 3 delineated by cysteines 256 and 265; and
- 20 9) potential protease processing sites at 2-3, 8-9, 23-24, 33-34, 36-37, 45-46, 48-49, 62-63, 66-67, 86-87, 110-111, 123-124, 134-135, 142-143, 272-273, 278-279 and 285-286;

Analogous regions in HRG- β 1 may be determined by reference to its' sequence. The analogous HRG- β 1 amino acids may be mutated or modified as discussed above for HRG- α . Analogous regions in HRG- β 2 may also be determined by reference to its' sequence. The analogous HRG- β 2 amino acids may be mutated or modified as discussed above for HRG- α or HRG- β 1. Analogous regions in HRG- β 3 may be determined by reference to its' sequence. Further, the analogous HRG- β 3 amino acids may be mutated or modified as discussed above for HRG- α , HRG- β 1, or HRG- β 2.

Another HRG variant is γ -HRG (or gamma-heregulin). γ -HRG is any polypeptide sequence that possesses at least one biological property of native sequence γ -HRG having SEQ ID NO:11. The biological property of this variant is the same as for HRG noted above. This variant encompasses not only the polypeptide isolated from a native γ -HRG source such as human MDA-MB-175 cells or from another source, such as another animal species, but also the polypeptide prepared by recombinant or synthetic methods. It also includes variant forms including functional derivatives, allelic variants, naturally occurring isoforms and analogues thereof. Sometimes the γ -HRG is "native γ -HRG" which refers to endogenous γ -HRG polypeptide which has been isolated from a mammal. The γ -HRG can also be "native sequence γ -HRG" insofar as it has the same amino acid sequence as a native γ -HRG (e.g. human γ -HRG shown in Fig. 7A-7C). Amino acid sequence variants of the native sequence are prepared by introducing appropriate nucleotide changes into the native sequence DNA, or by *in vitro* synthesis of the desired polypeptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence shown for the human protein in Fig. 7A-7C as generally described above for other HRG. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the native sequence, such as changing the number or position of O-linked

glycosylation sites.

Another variant is the polypeptide referred to as sensory and motor neuron derived factor (SMDF) whose nucleic acid and amino acid sequences (SEQ ID NOS:13 and 14) are shown in Fig. 8 which can be prepared as described in WO 96/15244. The SMDF polypeptides of the invention exhibit the properties of binding to the HER2/HER3 receptors and stimulating epithelial cell growth and differentiation in a manner similar to HRG polypeptides discussed above. Amino acid sequence variants of native sequence SMDF are prepared by introducing appropriate nucleotide changes into the native sequence SMDF DNA, or by *in vitro* synthesis of the desired SMDF polypeptide as observed generally above for other HRG. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence shown for human SMDF in Fig. 8. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the native sequence SMDF, such as changing the number or position of O-linked glycosylation sites.

Additional variants include polypeptides in which the variant has an amino acid substitution at a selected residue corresponding to a residue of 645-amino acid native human heregulin- β 1 selected from:

S177, H178, L179, V180, K181, E184, E186,
K187, T188, V191, N192, G193, G194, E195,
M198, V199, K200, D201, N204, P205, S206,
R207, Y208, L209, K211, P213, N214, E215,
T217, G218, D219, Q222, N223, Y224, S228, and F229.

In a variation of this embodiment, the amino acid substitution is not a replacement of the selected residue with an epidermal growth factor (EGF) residue corresponding to the selected residue.

Other heregulin- β 1 variants include an amino acid substitution selected from:

S177W; H178S, E, R, or A; V180Q, I or E;
K181P or A; A183G; E184V, W, K, R, G, or N;
K185E, S, Q, or G; E186R; K187E or A; T188Q;
E195Q; F197Y; M198R or K; K200R; D201T or I;
P205T or Y; S206K, H, G, P, or R; R207Y;
Y208R or L; L209M or G; K211R; P213S, T, N, or K;
N214L, K, S, or E; F216M; N223H or W; and M226I.

In a variation of this embodiment, the heregulin variant includes sets of amino acid substitutions selected from this group. Some heregulin variants of the invention having sets of amino acid substitutions exhibit at least a 50-fold increase in HER3 receptor affinity, which is also accompanied by an increase in HER4 receptor affinity. Specific variants include:

A183G, E184W, K185D, E186R, K187E, T188G, M226I;
A183D, E184K, K185S, E186R, K187E, T188G, M226I;
F197Y, M198K, K200R, D201I, M226I;
P205Y, S206G, R207Y, Y208L, L209M;
P205Y, S206R, R207Y, Y208R, L209M, M226I;
P205T, S206H, R207Y, Y208R, L209M;
P205T, S206K, R207Y, Y208R, L209G;
N223W, M226I;
N223H, M226I;
S177W, H178E, K181P, A183G, E184W, K185D, E186R, K187E, T188G, M226I;
P205Y, S206G, R207Y, Y208L, L209M, M226I;

A183G, K185E, E186R, K187E, T188G, F197Y, M198R, D201T;
 A183G, K185E, E186R, K187E, T188G, P205Y, S206G, R207Y, Y208L, L209M;
 A183G, K185E, E186R, K187E, T188G, F197Y, M198R, D201T, P205Y, S206G, R207Y, Y208L, L209M;
 A183G, K185E, E186R, K187E, T188G, M226I;
 5 F197Y, M198R, D201T, P205Y, S206G, R207Y, Y208L, L209M;
 F197Y, M198R, D201T, P205Y, S206G, R207Y, Y208L, L209M, M226I;
 F197Y, M198R, D201T, M226I;
 A183G, K185E, E186R, K187E, T188G, F197Y, M198R, D201T, M226I;
 A183G, K185E, E186R, K187E, T188G, P205Y, S206G, R207Y, Y208L, L209M, M226I;
 10 A183G, K185E, E186R, K187E, T188G, F197Y, M198R, D201T, P205Y, S206G, R207Y, Y208L, L209M,
 M226I;
 F197Y, M198R, D201T, P205Y, S206G, R207Y, Y208L, L209M, N223H, M226I; and
 A183G, K185E, E186R, K187E, T188G, F197Y, M198R, D201T, P205Y, S206G, R207Y, Y208L, L209M,
 N223H, M226I.

15 In addition to including one or more of the amino acid substitutions disclosed herein, the heregulin
 variant can have one or more other modifications, such as an amino acid substitution, an insertion of at least
 one amino acid, a deletion of at least one amino acid, or a chemical modification. For example, the invention
 provides a heregulin variant that is a fragment. In a variation of this embodiment, the fragment includes
 residues corresponding to a portion of human heregulin- β 1 extending from about residue 175 to about
 20 residue 230 (i.e., the EGF-like domain). For example, the fragment can extend from residue 177 to residue
 244 and may be prepared by recombinant techniques (rHRG β 1-177-244).

DNA encoding amino acid sequence variants of HRG is prepared by a variety of methods known in
 the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally
 occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed)
 25 mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant
 version of HRG. These techniques may utilize HRG nucleic acid (DNA or RNA), or nucleic acid
 complementary to HRG nucleic acid.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion,
 and insertion variants of HRG DNA. This technique is well known in the art as described by Adelman *et al.*,
 30 *DNA*, 2: 183 (1983). Briefly, HRG DNA is altered by hybridizing an oligonucleotide encoding the desired
 mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage
 containing the unaltered or native DNA sequence of HRG. After hybridization, a DNA polymerase is used
 to synthesize an entire second complementary strand of the template that will thus incorporate the
 oligonucleotide primer, and will code for the selected alteration in HRG DNA.

35 Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal
 oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either
 side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize
 properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using
 techniques known in the art such as that described by Crea *et al.* (*Proc. Natl. Acad. Sci. USA*, 75:
 40 5765, 1978).

Single-stranded DNA template may also be generated by denaturing double-stranded plasmid (or
 other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example),
 the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A
 45 DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to synthesize
 the complementary strand of the template using the oligonucleotide as a primer for synthesis. A

heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of HRG, and the other strand (the original template) encodes the native, unaltered sequence of HRG. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with ³²P-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: the single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(aS) (which can be obtained from Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion. After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with *ExoIII* nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101, as described above.

DNA encoding HRG mutants with more than one amino acid to be substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.

In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

PCR mutagenesis is also suitable for making amino acid variants of HRG. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see Erlich, *supra*, the chapter by R. Higuchi, p. 61-70). When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the

primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

In a specific example of PCR mutagenesis, template plasmid DNA (1mg) is linearized by digestion with a restriction endonuclease that has a unique recognition site in the plasmid DNA outside of the region to be amplified. Of this material, 100 ng is added to a PCR mixture containing PCR buffer, which contains the four deoxynucleotide tri-phosphates and is included in the GENEAMP kits (obtained from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA), and 25 pmole of each oligonucleotide primer, to a final volume of 50 ml. The reaction mixture is overlaid with 35 ml mineral oil. The reaction is denatured for 5 minutes at 100°C, placed briefly on ice, and then 1 ml *Thermus aquaticus* (Taq) DNA polymerase (5 units/ml, purchased from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA) is added below the mineral oil layer. The reaction mixture is then inserted into a DNA Thermal Cycler (purchased from Perkin-Elmer Cetus) programmed as follows:

2 min. 55°C,
30 sec. 72°C, then 19 cycles of the following:
30 sec. 94°C,
30 sec. 55°C, and
30 sec. 72°C.

At the end of the program, the reaction vial is removed from the thermal cycler and the aqueous phase transferred to a new vial, extracted with phenol/chloroform (50:50:vol), and ethanol precipitated, and the DNA is recovered by standard procedures. This material is subsequently subjected to the appropriate treatments for insertion into a vector.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells *et al.* (*Gene*, 34: 315, 1985). The starting material is the plasmid (or other vector) comprising HRG DNA to be mutated. The codon(s) in HRG DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in HRG DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated HRG DNA sequence.

C. Insertion of DNA into a Cloning Vehicle

The cDNA or genomic DNA encoding native or variant HRG is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of

the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal Sequence Component

In general, the signal sequence may be a component of the vector, or it may be a part of HRG DNA that is inserted into the vector. The native HRG DNA is believed to encode a signal sequence at the amino terminus (5' end of the DNA encoding HRG) of the polypeptide that is cleaved during post-translational processing of the polypeptide to form the mature HRG polypeptide ligand that binds to the HER2/HER3 receptor, although a conventional signal structure is not apparent. Native HRG is, secreted from the cell but remains lodged in the membrane because it contains a transmembrane domain and a cytoplasmic region in the carboxyl terminal region of the polypeptide. Thus, in a secreted, soluble version of HRG the carboxyl terminal domain of the molecule, including the transmembrane domain, is ordinarily deleted. This truncated variant HRG polypeptide may be secreted from the cell, provided that the DNA encoding the truncated variant encodes a signal sequence recognized by the host.

HRG of this invention may be expressed not only directly, but also as a fusion with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-and/or C-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of HRG DNA that is inserted into the vector. Included within the scope of this invention are HRG with the native signal sequence deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be one that is recognized and processed, i.e., cleaved by a signal peptidase, by the host cell. For prokaryotic host cells that do not recognize and process the native HRG signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native HRG signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the native signal sequence is satisfactory, although other mammalian signal sequences may be suitable.

(ii) Origin of Replication Component

Both expression and cloning vectors generally contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2m plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of HRG DNA. However, the recovery of genomic DNA

encoding HRG is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise HRG DNA. DNA can be amplified by PCR and directly transfected into the host cells without any replication component.

(iii) Selection Gene Component

5 Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene
10 encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al.*, *J. Molec. Appl. Genet.* 1: 327,1982), mycophenolic acid (Mulligan *et al.*, *Science* 209: 1422,1980) or hygromycin (Sugden *et al.*, *Mol. Cell. Biol.* 5: 410-413,1985). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the
20 identification of cells competent to take up HRG nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA
25 that encodes HRG. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of HRG are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR.
30 An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77: 4216, 1980. The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding HRG. This amplification technique can
35 be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding HRG, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium
40 containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418 (see U.S. Pat. No. 4,965,199).

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, *Nature*, 282: 39, 1979; Kingsman *et al.*, *Gene*, 7: 141, 1979; or Tschemper *et al.*, *Gene*, 10: 157, 1980). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to
45 grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 85: 12, 1977). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting

transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

(iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to HRG nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as HRG to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding HRG by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native HRG promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of HRG DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed HRG as compared to the native HRG promoter.

Promoters suitable for use with prokaryotic hosts include the b-lactamase and lactose promoter systems (Chang *et al.*, *Nature*, 275: 615, 1978; and Goeddel *et al.*, *Nature* 281: 544, 1979), alkaline phosphatase, a tryptophan (*trp*) promoter system (Goeddel, *Nucleic Acids Res.*, 8: 4057, 1980 and EP 36,776), tPA (U.S. 5,641,655) and hybrid promoters such as the *tac* promoter (deBoer *et al.*, *Proc. Natl. Acad. Sci. USA* 80: 21-25, 1983). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding HRG (Siebenlist *et al.*, *Cell* 20: 269, 1980) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding HRG.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, *J. Biol. Chem.*, 255: 2073, 1980) or other glycolytic enzymes (Hess *et al.*, *J. Adv. Enzyme Reg.* 7: 149, 1968; and Holland, *Biochemistry* 17: 4900, 1978), such as enolase, glyceraldehyde 3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytocrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman *et al.*, EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into mammalian expression vectors.

HRG gene transcription from vectors in mammalian host cells may be controlled by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504, published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian

promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with HRG sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction
 5 fragment that also contains the SV40 viral origin of replication (Fiers *et al.*, *Nature*, 273:113 (1978); Mulligan and Berg, *Science*, 209: 1422-1427 (1980); Pavlakis *et al.*, *Proc. Natl. Acad. Sci. USA*, 78: 7398-7402 (1981)). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a
 HindIII E restriction fragment (Greenaway *et al.*, *Gene*, 18: 355-360 (1982)). A system for expressing DNA
 in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. 4,419,446. A
 10 modification of this system is described in U.S. 4,601,978. See also Gray *et al.*, *Nature*, 295: 503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes *et al.*, *Nature*, 297: 598-601 (1982) on expression of human b-interferon cDNA in mouse cells under the control of a thymidine
 kinase promoter from herpes simplex virus; Canaani and Berg, *Proc. Natl. Acad. Sci. USA*, 79: 5166-5170 (1982) on expression of the human interferon b1 gene in cultured mouse and rabbit cells; and Gorman *et al.*,
 15 *Proc. Natl. Acad. Sci. USA*, 79: 6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(v) Enhancer Element Component

Transcription of a DNA encoding HRG of this invention by higher eukaryotes is often increased by
 20 inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins *et al.*, *Proc. Natl. Acad. Sci. USA*, 78: 993, 1981) and 3' (Lusky *et al.*, *Mol. Cell Bio.*, 3: 1108, 1983) to the transcription unit, within an intron (Banerji *et al.*, *Cell*, 33: 729, 1983) as well as within the coding sequence itself (Osborne *et al.*, *Mol. Cell Bio.*, 4: 1293, 1984).
 25 Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, a-fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers (see also Yaniv, *Nature*, 297: 17-18 (1982)) on enhancing elements for activation of eukaryotic promoters.
 30 The enhancer may be spliced into the vector at a position 5' or 3' to HRG DNA, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or
 nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination
 35 of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding HRG. The 3' untranslated regions also include transcription termination sites.

Construction of suitable vectors containing one or more of the above listed components the desired
 40 coding and control sequences employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to
 transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or
 tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by
 45 restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, *Nucleic Acids Res.* 9: 309 (1981) or by the method of Maxam *et al.*, *Methods in Enzymology* 65: 499 (1980).

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding HRG. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of HRG that have HRG-like activity. Such a transient expression system is described in U.S. 5,024,939.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of HRG in recombinant vertebrate cell culture are described in Gething *et al.*, *Nature* 293: 620-625, 1981; Mantei *et al.*, *Nature*, 281: 40-46, 1979; Levinson *et al.*, EP 117,060 and EP 117,058. A particularly useful expression plasmid for mammalian cell culture expression of HRG is pRK5 (EP pub. no. 307,247).

15 **D. Selection and Transformation of Host Cells**

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, *Bacilli* such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescans*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* x1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, *in vitro* methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for HRG-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* (Beach and Nurse, *Nature*, 290: 140 (1981); EP 139,383, published May 2, 1985), *Kluyveromyces* hosts (U.S.S.N. 4,943,529) such as, e.g., *K. lactis* (Louvencourt *et al.*, *J. Bacteriol.*, 737 (1983); *K. fragilis*, *K. bulgaricus*, *K. thermotolerans*, and *K. marxianus*, *yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070), Sreekrishna *et al.*, *J. Basic Microbiol.*, 28: 265-278 (1988); *Candida*, *Trichoderma reesia* (EP 244,234); *Neurospora crassa* (Case *et al.*, *Proc. Natl. Acad. Sci. USA*, 76: 5259-5263 (1979), and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolyocladium* (WO 91/00357, published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, *Biochem. Biophys. Res. Commun.*, 112: 284-289 (1983); Tilburn *et al.*, *Gene*, 26: 205-221 (1983); Yelton *et al.*, *Proc. Natl. Acad. Sci. USA*, 81: 1470-1474 (1984) and *A. niger* (Kelly and Hynes, *EMBO J.*, 4: 475-479 (1985)).

Suitable host cells for the expression of glycosylated HRG polypeptide are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have been identified (see, e.g., Luckow *et al.*, *Bio/Technology*, 6: 47-55 (1988); Miller *et al.*, in *Genetic Engineering*, Setlow, J.K. *et al.*, eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda *et al.*, *Nature*, 315: 592-594 (1985)). A variety of such viral strains are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain HRG DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding HRG is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express HRG DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences (Depicker *et al.*, *J. Mol. Appl. Gen.*, 1: 561 (1982)). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue (see EP 321,196, published 21 June 1989).

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, *J. Gen Virol.*, 36: 59, 1977); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77: 4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.*, 23: 243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, *Annals N.Y. Acad. Sci.*, 383: 44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2). Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO_4 and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integration. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al.*, *supra*, is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw *et al.*, *Gene*, 23: 315 (1983) and WO 89/05859, published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30-16.37 of Sambrook *et al.*, *supra*, is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. 4,399,216, issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, *J. Bact.*, 130: 946 (1977) and Hsiao *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 76: 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used.

E. Culturing the Host Cells

Prokaryotic cells used to produce HRG polypeptide of this invention are cultured in suitable media as described generally in Sambrook *et al.*, *supra*.

The mammalian host cells used to produce HRG of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.*, 58: 44 (1979), Barnes and Sato, *Anal. Biochem.*, 102: 255 (1980), U.S. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. Re. 30,985; or U.S. 5,122,469 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in *in vitro* culture as well as cells that are within a host animal.

It is further envisioned that HRG of this invention may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding HRG currently in use in the field. For example, a powerful promoter/enhancer element, a suppresser, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired HRG. The control element does not encode HRG of this invention, but the DNA is present in the host cell genome. One next screens for cells making HRG of this invention, or increased or decreased levels of expression, as desired.

F. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA*, 77: 5201-5205 (1980)), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ³²P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled where the labels are usually visually detectable such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu *et al.*, *Am. J. Clin. Path.*, 75: 734-738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be

prepared against a native HRG polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further below.

G. Purification of The Heregulin Polypeptides

HRG is recovered from a cellular membrane fraction. Alternatively, a proteolytically cleaved or a truncated expressed soluble HRG fragment or subdomain are recovered from the culture medium as a soluble polypeptide. A HRG is recovered from host cell lysates when directly expressed without a secretory signal.

When HRG is expressed in a recombinant cell other than one of human origin, HRG is completely free of proteins or polypeptides of human origin. However, it is desirable to purify HRG from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to HRG. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. HRG is then be purified from both the soluble protein fraction (requiring the presence of a protease) and from the membrane fraction of the culture lysate, depending on whether HRG is membrane bound. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica, heparin SEPHAROSE or on a cation exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; and gel filtration using, for example, SEPHADEX G-75.

HRG variants in which residues have been deleted, inserted or substituted are recovered in the same fashion as the native HRG, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a HRG fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion. Immunoaffinity columns such as a rabbit polyclonal anti-HRG column can be employed to absorb HRG variant by binding it to at least one remaining immune epitope. A protease inhibitor such as phenylmethylsulfonylfluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native HRG may require modification to account for changes in the character of HRG variants or upon expression in recombinant cell culture.

H. Covalent Modifications of HRG

Covalent modifications of HRG polypeptides are included within the scope of this invention. Both native HRG and amino acid sequence variants of HRG optionally are covalently modified. One type of covalent modification included within the scope of this invention is a HRG polypeptide fragment. HRG fragments, such as HRG-GDF, having up to about 40 amino acid residues are conveniently prepared by chemical synthesis, or by enzymatic or chemical cleavage of the full-length HRG polypeptide or HRG variant polypeptide. Other types of covalent modifications of HRG or fragments thereof are introduced into the molecule by reacting targeted amino acid residues of HRG or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteiny residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteiny residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl picolinimate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ($R'-N=C=N-R'$), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginy and glutaminy residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking HRG to a water-insoluble support matrix or surface for use in a method for purifying anti-HRG antibodies, and vice versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-((p-azidophenyl)dithio)propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminy and asparaginy residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

HRG optionally is fused with a polypeptide heterologous to HRG. The heterologous polypeptide optionally is an anchor sequence such as that found in a phage coat protein such as M13 gene III or gene VIII proteins. These heterologous polypeptides can be covalently coupled to HRG polypeptide through side chains or through the terminal residues.

HRG may also be covalently modified by altering its native glycosylation pattern. One or more carbohydrate substituents in these embodiments, are modified by adding, removing or varying the monosaccharide components at a given site, or by modifying residues in HRG as that glycosylation sites are added or deleted.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tri-peptide sequences

asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tri-peptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose, to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Glycosylation sites are added to HRG by altering its amino acid sequence to contain one or more of the above-described tri-peptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to HRG (for O-linked glycosylation sites). For ease, HRG is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding HRG at preselected bases such that codons are generated that will translate into the desired amino acids.

Chemical or enzymatic coupling of glycosides to HRG increases the number of carbohydrate substituents. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that is capable of N- and O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330, published 11 September 1987, and in Aplin and Wriston (*CRC Crit. Rev. Biochem.*, pp. 259-306 (1981)).

Carbohydrate moieties present on an HRG also are removed chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin *et al.* (*Arch. Biochem. Biophys.*, 259:52 (1987)) and by Edge *et al.* (*Anal. Biochem.*, 118:131 (1981)). Carbohydrate moieties are removed from HRG by a variety of endo- and exo- glycosidases as described by Thotakura *et al.* (*Meth. Enzymol.*, 138:350 (1987)).

Glycosylation also is suppressed by tunicamycin as described by Duskin *et al.* (*J. Biol. Chem.*, 257:3105 (1982)). Tunicamycin blocks the formation of protein-N-glycoside linkages.

HRG may also be modified by linking HRG to various nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

One preferred way to increase the *in vivo* circulating half life of non-membrane bound HRG is to conjugate it to a polymer that confers extended half-life, such as polyethylene glycol (PEG). (Maxfield, *et al.*, *Polymer* 16,505-509 (1975); Bailey, F. E., *et al.*, in *Nonionic Surfactants* (Schick, M. J., ed.) pp.794-821, 1967); (Abuchowski, A. *et al.*, *J. Biol. Chem.* 252, 3582-3586, 1977; Abuchowski, A. *et al.*, *Cancer Biochem. Biophys.* 7, 175-186, 1984); (Katre, N.V. *et al.*, *Proc. Natl. Acad. Sci.*, 84, 1487-1491, 1987; Goodson, R. *et al. Bio Technology*, 8, 343-346, 1990). Conjugation to PEG also has been reported to have reduced immunogenicity and toxicity (Abuchowski, A. *et al.*, *J. Biol. Chem.*, 252, 3578-3581, 1977).

HRG may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences*, 16th edition, Osol, A., Ed., (1980).

Those skilled in the art will be capable of screening variants in order to select the optimal variant for the purpose intended. For example, a change in the immunological character of HRG, such as a change in affinity for a given antigen or for the HER2 receptor, is measured by a competitive-type immunoassay using

a standard or control such as a native HRG (in particular native HRG-GFD). Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, stability in recombinant cell culture or in plasma, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

5 I. Heregulin Antibody Preparation

The antibodies of this invention are obtained by routine screening and include polyclonal antibodies, monoclonal antibodies and fragments thereof.

10 Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}^1\text{N}=\text{C}=\text{NR}$, where R and R^1 are different alkyl groups.

15 Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed
20 for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*,
25 *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable
30 fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine,
35 aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOP-21 and
40 M.C.-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York,
45 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

5 The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable
10 culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-SEPHAROSE, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

15 DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that
20 do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

Hybridoma cell lines producing antibodies are identified by screening the culture supernatants for antibody which binds to HER2/HER3 receptors. This is routinely accomplished by conventional immunoassays using soluble receptor preparations or by FACS using cell-bound receptor and labeled
25 candidate antibody. Agonist antibodies are preferably antibodies which stimulate autophosphorylation in the HRG tyrosine autophosphorylation assay described above.

The hybrid cell lines can be maintained in culture *in vitro* in cell culture media. The cell lines of this invention can be selected and/or maintained in a composition comprising the continuous cell line in hypoxanthine-aminopterin thymidine (HAT) medium. In fact, once the hybridoma cell line is established, it
30 can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody. The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, ion exchange chromatography, affinity chromatography, or the like. The
35 antibodies described herein are also recovered from hybridoma cell cultures by conventional methods for purification of IgG or IgM as the case may be that heretofore have been used to purify these immunoglobulins from pooled plasma, e.g., ethanol or polyethylene glycol precipitation procedures.

Human antibodies may be used and are preferable. Such antibodies can be obtained by using human hybridomas (Cote *et al.*, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985)).
40 Chimeric antibodies, Cabilly *et al.*, U.S. 4,816,567, (Morrison *et al.*, *Proc. Natl. Acad. Sci.*, 81:6851 (1984); Neuberger *et al.*, *Nature* 312:604 (1984); Takeda *et al.*, *Nature* 314:452 (1985)) containing a murine anti-HER2/HER3 variable region and a human constant region of appropriate biological activity (such as ability to activate human complement and mediate ADCC) are within the scope of this invention, as are humanized antibodies produced by conventional CDR-grafting methods (Riechmann *et al.*, *Nature* 332:333-327(1988);
45 EP 0328404 A1; EP 02394000 A2).

Techniques for creating recombinant DNA versions of the antigen-binding regions of antibody molecules (Fab or variable regions fragments) which bypass the generation of monoclonal antibodies are also

encompassed within the practice of this invention. One extracts antibody-specific messenger RNA molecules from immune system cells taken from an immunized subject, transcribes these into complementary DNA (cDNA), and clones the cDNA into a bacterial expression system and selects for the desired binding characteristic. The Scripps/Stratagene method uses a bacteriophage lambda vector system containing a leader sequence that causes the expressed Fab protein to migrate to the periplasmic space (between the bacterial cell membrane and the cell wall) or to be secreted. One can rapidly generate and screen great numbers of functional Fab fragments to identify those which bind the receptors with the desired characteristics. Alternatively, the antibodies can be prepared by the phage display techniques described in Hoogenboom, *Tibtech* February 1997 (vol 15); Neri *et al.*, *Cell Biophysics* 27:47-61 (1995); Winter *et al.*, *Annu. Rev. Immunol.* 12:433-55 (1994); and Soderlind *et al.*, *Immunol. Rev.* 130:109-124 (1992) and the references described therein as well as the monovalent phage display technique described in Lowman *et al.*, *Biochem.* 30:10832-10838 (1991).

2. Therapeutic compositions, administration and use of Heregulins and Agonist Antibodies

The HRG are used in the present invention to induce epithelial cell growth, for example lung epithelial cell growth, proliferation and differentiation, and to increase the production of surfactant protein A by lung cells. These effects allow treatment of disease states associated with tissue damage, for example, chronic obstructive pulmonary disease (COPD) including subtypes thereof such as chronic bronchitis, emphysema, asthma, etc., neonatal pulmonary diseases including neonatal respiratory distress syndrome, meconium aspiration syndrome, chronic lung disease of the neonate, congenital diaphragmatic hernia, etc., acute lung injuries including smoke or chemical inhalation, pneumonitis due to aspiration, radiation, etc., near drowning, cystic fibrosis and other epithelial cell trauma diseases, including injuries associated with surgical wounds and resections, ulcers, lesions, and tissue tears, with the method of the invention.

A preferred indication for treatment with the method of the invention is the treatment of COPD. COPD is a spectrum of chronic inflammatory respiratory diseases characterized by cough, sputum, dyspnea, airflow limitation and impaired gas exchange. COPD is common in older populations and presents a pattern of gradually declining lung function. Typically, a patient will exhibit a chronic cough with clear sputum which worsens to a cough with thick sputum and accompanying poor air exchange. These conditions frequently lead to heart disease and death. Many persons with COPD will have chronic bronchitis together with emphysema. The present invention is particularly important because it halts, slows and/or reverses the lung destruction process in COPD patients. In this action, the method of the invention is very different from typical treatments for COPD in which the symptoms are treated, but not the underlying destruction of lung cell tissue and function.

The method of the invention may, however, be combined with or administered together with other therapies for treatment of lung disease such as COPD. For example, the method of this invention can be used together with the administration of an anticholinergic bronchodilator such as ipratropium bromide (ATROVENT available from Boehringer Ingelheim) or tiotropium, a beta adrenergic receptor agonist such as albuterol (PROVENTIL available from Schering) or salmeterol, steroids such as prednisone, retinoic acid, phosphodiesterase inhibitors, endothelin antagonists, metalloproteinase inhibitors, elastase inhibitors, free radical inhibitors, serine proteinase inhibitors, neutrophil elastase inhibitors, pulmonary surfactant compositions such as beractant (SURVANTA available from Ross Labs.), PDGF, FGF, EGF, growth hormone or other protein growth factors, etc. or combinations thereof. The relative amount of the HRG and the additional compound(s) can be readily determined by a physician with regard to the individual symptoms of the patient. It is anticipated that these compositions and the relative amounts of the components therein will be varied as necessary to address the specific needs of a patient and will be monitored and adjusted using conventional physiochemical and medical tests for lung function.

Therapeutic formulations of HRG or agonist antibody are prepared for storage by mixing the HRG protein having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (*Remington's Pharmaceutical Sciences, supra*), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN, PLURONICS or polyethylene glycol (PEG).

HRG or agonist antibody to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The HRG or antibody ordinarily will be stored in lyophilized form or in solution.

Therapeutic HRG or antibody compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of HRG or antibody administration is in accord with known methods, e.g., injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, powder or liquid aerosol administration to the nose or lung or intralesional routes, or by sustained release systems as noted below. The HRG ligand may be administered continuously by infusion or by bolus injection. An agonist antibody is preferably administered in the same fashion, or by administration into the blood stream or lymph.

The HRG, HRG variant or fragment and agonist antibodies may be spray dried or spray freeze dried using known techniques (Yeo et al, *Biotech. and Bioeng.*, 41:341-346 (1993); Gombotz et al, PCT/US90/02421).

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer et al., *J. Biomed. Mater. Res.*, 15: 167-277 (1981) and Langer, *Chem. Tech.*, 12: 98-105 (1982) or poly(vinylalcohol)), polylactides (U.S. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., *Biopolymers*, 22: 547-556 (1983)), non-degradable ethylene-vinyl acetate (Langer et al., *supra*), degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988). While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release HRG or antibody compositions also include liposomally entrapped HRG or antibody. Liposomes containing HRG or antibody are prepared by methods known *per se*: DE 3,218,121; Epstein et al., *Proc. Natl. Acad. Sci. USA*, 82: 3688-3692 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA*,

77: 4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal HRG therapy. Liposomes with enhanced circulation time are disclosed in U.S. patent 5,013,556.

An effective amount of HRG or antibody to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Also, the amount of HRG polypeptide will generally be less than the amount of an agonist antibody. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 µg/kg to about 1 mg/kg and up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer HRG or antibody until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays, for example, surfactant protein A production.

In a further embodiment, epithelial cells may be obtained or isolated from a mammalian tissue to obtain a normal epithelial cell sample using techniques well known in the art (biopsy, etc.). This sample may then be treated with a heregulin protein in order to induce epithelial cell growth and/or proliferation in the sample thereby expanding the population of primary epithelial cells. Typically, heregulin will be added to the *in vitro* epithelial cell culture at a concentration of about 0.1 to about 100 nM preferably 1-50 nM. If desired, the primary epithelial cells may be cultured *in vitro* for several generations in order to sufficiently expand the epithelial cell population. The epithelial cells are cultured under conditions suitable for mammalian cell culture as discussed above. After expansion, the expanded sample is reintroduced into the mammal for the purpose of re-epithelializing the mammalian tissue. For example, lung epithelial cells isolated from a patient having emphysema or chronic obstructive pulmonary disease may be obtained, expanded and reintroduced into the lung in order to more quickly re-epithelialize the damaged lung tissue thereby reestablishing lung function. The expanded cells may be reintroduced into the lung by aspiration or intubation using methods well known in the art.

The methods and procedures described herein with respect to HRG-α or HRG in general may be applied similarly to other HRG such as HRG-β1, HRG-β2 and HRG-β3 and to variants thereof, as well as to the antibodies. The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1 - Preparation of Heregulins

(a) Heregulins HRG-α, HRG-β1, HRG-β2, HRG-β2-like, and HRG-β3 were isolated, cloned, expressed and isolated from the cell culture medium as described in U.S. 5,367,060.

(b) SMDF polypeptides are prepared as described in WO 96/15244.

(c) γ-HRG polypeptide was prepared and characterized as described below.

Reagents: The EGF-like domain of HRGβ1₍₁₇₇₋₂₄₄₎ was expressed in *E. coli*, purified and radioiodinated as described previously (Sliwkowski *et al. J. Biol. Chem.* 269:14661-14665 (1994)). The anti-HER2 monoclonal antibodies 2C4 and 4D5 have been described elsewhere (Fendly *et al. Cancer Research* 50:1550-1558 (1990)).

HER3 and HER4-immunoadhesins: A unique M1 I site was engineered into a plasmid expressing human IgG heavy chain at the region encoding the hinge domain of the immunoglobulin. M1 I sites were also engineered into a set of HER expression plasmids at the region encoding the ECD/TM junctions of these receptors. All mutageneses were done using the Kunkel method (Kunkel, T., *Proc. Natl. Acad. Sci. U.S.A.* 82:488 (1985)). The M1 I sites were utilized to make the appropriate HER-IgG fusion constructs. The fusion junctions of the various HER-IgG chimeras were: for HER2, E⁶⁴⁶_{HER2-(TR)-DKTH²²⁴}_{VH}; for HER3,

L⁶³⁶ HER3-(TR)-DKTH²²⁴_{VH}; for HER4, G⁶⁴⁰ HER4-(TR)-DKTH²²⁴_{VH}. The conserved TR sequence is derived from the M1 I site. The final expression constructs were in a pRK-type plasmid backbone wherein eukaryotic expression is driven by a CMV promoter (Gorman *et al.*, *DNA Prot. Eng. Tech.* 2:3-10 (1990)).

To obtain protein for *in vitro* experiments, adherent HEK-293 cells were transfected with the appropriate expression plasmids using standard calcium phosphate methods (Gorman *et al.*, *supra* and Huang *et al.*, *Nucleic Acids Res.* 18:937-947 (1990)). Serum-containing media was replaced with serum-free media 15 hours post-transfection and the transfected cells incubated for 5-7 days. The resulting conditioned media was harvested and passed through Protein A columns (1 mL Pharmacia HiTrap™). Purified IgG fusions were eluted with 0.1 M citric acid (pH 4.2) into tubes containing 1 M Tris pH 9.0. The eluted proteins were subsequently dialyzed against PBS and concentrated using Centri-prep-30 filters (Amicon). Glycerol was added to a final concentration of 25% and the material stored at -20 C. Concentrations of material were determined via a Fc-ELISA.

Cell Culture: Human breast cancer cell lines MDA-MB-175, MDA-MB-231, SK-BR-3 and MCF7 were obtained from the American Type Culture Collection and maintained in a 50:50 mixture of F12 Ham's and Dulbecco's modified Eagle medium (DMEM), supplemented with 10% heat inactivated FBS, 2 mM glutamine and 10% penicillin-streptomycin.

Generation and Characterization of cDNA Library: Total RNA was purified from MDA-MB-175 cells using the guanidinium isothiocyanate-cesium chloride procedure (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, (1989)). Poly (A)⁺ RNA was isolated using oligo (dT) Dynabeads (DYNAL) as recommended by the supplier. First and second strand syntheses were performed using a Gibco BRL cDNA synthesis kit. λ gt10 cDNA recombinants were generated when a cDNA cloning system from Amersham was used. *In vitro* packaging was performed using Gigapack II packaging extract (Stratagene). PstI-XhoI HRG β 3 cDNA fragment (nt 144-618) was labeled by random priming and 1 x 10⁶ plaques were screened. Positive clones were confirmed and purified by secondary and tertiary screening. Phage DNA was isolated as a BamHI fragment and subcloned into the corresponding site of pBluescript SK⁺. Clone 5 was completely sequenced using the Sequenase version 2.0 DNA sequencing kit (United States Biochemicals, Inc.). Both strands were sequenced.

Bacterial Expression System: A cDNA fragment of clone 5 (nt 1690- 2722) was subcloned into the pET-32 TRX fusion vector (Novagen). This BglII-BglII fragment was inserted into the BamHI site of the pET32a plasmid. The trxy-HRG (amino acids 455-768) protein expression in *E.coli* was induced as recommended by the supplier.

Purification of Recombinant γ -HRG: *E.coli* cells expressing trxy-HRG were collected and suspended at 9 ml/g in 50 mM Tris HCL pH 8. Lysozyme was added to a final concentration of 0.2 mg/ml and the solution was stirred on ice for 1 hr. Dnase I (10 μ g/ml) and MgCl₂ (4 mM) were added. The solution was then sonicated for 30 min and cell pellets collected afterwards. The pellet fraction was dissolved at 250 ml/g in 6 M Gdn HCL, 0.1 M Tris HCL, pH 8.8. Solubilized proteins were sulfitylolyzed by adding 1/10 volume of 1 M Na₂SO₃ and 1/10 volume of 0.2 M Na₂S₄O₆. The reaction was allowed to proceed for 1.5 hours at room temperature and protein was purified by gel filtration chromatography using a High Load Superdex™ 75 prep grade column (Pharmacia). Refolding was initiated by the addition of 1 mM cysteine, and 10 mM methionine was added as an antioxidant and incubated overnight at room temperature. Protein concentration was determined by quantitative amino acid analysis.

Northern and Southern Hybridization: Total RNA was isolated by the method of Chomczynski *et al.* *Anal Biochem.* 162:156-159 (1987). Poly (A)⁺ was isolated using oligo d(T) cellulose columns (Qiagen) as recommended by the supplier. RNA was denatured and size fractioned in a 0.8% formaldehyde/ 1%

agarose gel and transferred onto nylon membrane (Hybond, Amersham). RNA was UV crosslinked (UV Stratalinker, Stratagene). Prehybridization was carried out at 42 C in 50 %formamide/ 1% SDS/ 1 M NaCl, 10 % dextran sulfate and 100 µg/ml herring sperm DNA for at least 2 hours. cDNA probes using either a restriction fragment with complementary sequence to the EGF-like domain of HRGβ3 or a KpnI-AvaII cDNA fragment encoding the unique sequence of γ-HRG (nt 1238-1868) were radiolabeled by random priming (Prime-It II, Stratagene). Hybridization was done in equal solution at 42°C containing the ³²P labeled fragments for 16 hr. Blots were washed several times with 2 x SSC/ 1% SDS at room temperature, washed with the same solution at 65°C for 20 min and finally washed with 0.2 x SSC/ 0.1% SDS at room temperature for 15 min. The blots were air dried and exposed to Du Pont Reflection™ film with intensifying screens at -80°C for 7-40 hours. Human multiple tissue Northern blots (Clontech) containing 2 µg poly (A)⁺ from spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocytes, heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas were hybridized with a radiolabeled γ-HRG cDNA probe (nt 841-1447) as recommended by the supplier.

MDA-MB-175 and MDA-MB-231 genomic DNA was isolated as described in Sambrook *et al*, *supra*. DNA was digested with different restriction enzymes, prior to transfer treated with 0.25 N HCl and transferred onto nylon membrane (Hybond, Amersham). BglII-NdeI cDNA fragment of γ-HRG (nt 1690-2351) was also radiolabeled by random priming and used as a hybridization probe. Prehybridization was carried out in 6 x SSC/ 5 x Denhardt's/ 0.75% SDS, 10% Dextran sulfate and 100 µg/ml herring sperm DNA at 68 C for 4 hours and hybridization with radiolabeled probe was done overnight. The same wash conditions as for Northern blots were used except a wash step with 0.2 x SSC/ 0.1% SDS at 68°C was added and detection was pursued as described above.

¹²⁵I-HRG Binding Assay: Binding assays were performed in Nunc breakapart strip wells. Plates were coated at 4°C overnight with 100 µl of 5 µg/ml goat-anti-human antibody (Boehringer Mannheim) in 50 mM carbonate buffer (pH 9.6). Plates were rinsed twice with wash buffer (PBS/ 0.05% Tween-20) and blocked with 100 µl 1% BSA/ PBS for 30 min. Buffer was removed and each well was incubated with 15 ng IgG fusion protein in 1% BSA/ PBS under vigorous shaking for 1.5 hours. Plates were rinsed three times with wash buffer and competitive binding was carried out by adding various amounts of γ-HRG and ¹²⁵I-HRGβ1 under vigorous shaking. After incubation for 1.5-2 hours, wells were rinsed three times with wash buffer, drained and individual wells were counted using a 100 Series Iso Data γ-counter.

Tyrosine Phosphorylation Assay: MCF7 cells were plated in 24 well plates at 1 x 10⁵ cells/ well in F12/DMEM containing 10% FBS. After 48 hours, cells were washed with serum free F12/DMEM and serum starved for 6 hours. Various concentrations of bacterial expressed truncated γ-HRG (*i.e.*, 0 pM, 22 pM, 66 pM, 200 pM and 600 pM trγ-HRG) or unpurified conditioned medium of MDA-MB-175 cells were prepared in binding buffer (0.1% BSA in F12/DMEM) and added to each well. After 8 min incubation at room temperature, media was carefully aspirated and reactions were stopped by adding 100 µl of sample buffer (5% SDS, 0.25% 2-mercaptoethanol, 25 mM Tris-HCL pH 6.8). 20 µl of each sample was size fractionated in a 4-12% gradient gel (Novex) and then electrophoretically transferred onto nitrocellulose membrane. Antiphosphotyrosine (4G10, from UBI, used at 1 µg/ml) immunoblots were developed and the predominant reactive band at M_r ~180 kDa was quantified by reflectance densitometry.

Production and characterization of conditioned medium from MDA-MB-175 cells. Cells were

seeded in T175 flasks and grown until reaching 70-80% confluency ($\sim 2.5 \times 10^7$ cells/ flask). Subsequently, cells were washed with PBS and grown in serum free F12/ DMEM medium for 3-4 days. Medium was then collected, filtered and concentrated using an ultrafiltration cell with YM10 Diaflo ultrafiltration membranes (Amicon). γ -HRG was visualized in conditioned medium of MDA-MB-175 cells by Western blot analysis under non reducing conditions. γ -HRG was partially purified by HPLC using a C4 reverse phase column. CHO expressed full length HRG β 1 (lane 1) and semi pure γ -HRG (lane 2) were electrophoresed, blot was probed with HER2/HER4 IgG heterodimers and Western blot was developed. A ~ 64 kDa band could be seen in the lane containing partial purified supernatant whereas CHO expressed full length HRG β 1 migrated as a 45 kDa protein.

Cell Proliferation Assay with Crystal Violet: Tumor cell lines were plated in 96 well plates at following densities: 2×10^4 cells/well for MDA-MB-175 and 1×10^4 cells/well for SK-BR-3. The media contained 1% FBS and cells were allowed to adhere for 2 hours. Monoclonal antibodies, immunoadhesions (10 μ g/ml) or media alone were added and the cells were incubated for 2 hours at 37°C. rHRG β 1₁₇₇₋₂₄₄ was added at a final concentration of 1 nM, or 100 nM for neutralising the immunoadhesion, and the cells were incubated for 4 days. Monolayers were washed with PBS and stained/fixed with 0.5% crystal violet. Plates were air dried, the dye was eluted with 0.1 M sodium citrate (pH 4.2) in ethanol (50:50) and the absorbance was measured at 540 nm.

Isolation and sequence analysis of γ -HRG: To characterize the heregulin transcript in MDA-MB-175 cells, a λ gt 10 cDNA library was constructed with mRNA derived from this cell line. The library was screened with a cDNA probe corresponding to the EGF-like domain and part of the N-terminal sequence of HRG β 3. Various clones were identified. One of the clones which appeared to contain the full length cDNA sequence was isolated and sequenced. Fig.7A-7D shows the nucleotide sequence and the predicted amino acid sequence of γ -HRG. The single open reading frame of 2303 bp starts with an ATG codon at nt 334. This start codon lies in a nucleotide sequence context, which is known to be a potential translation initiation site (Kozak, *Nucleic Acid Research* 15:8125-8148 (1987)). Several termination codons were found upstream of the initiation codon. The stop codon TAG at nt 2637 is followed by the 3' noncoding sequence, which is identical to other HRG isoform sequences and includes a polyadenylation signal followed by an A-rich region. The open reading frame encodes a protein of 768 amino acid residues with a calculated molecular mass of 84.2 kDa.

(d) The selection of HRG- β 1 variants containing residues corresponding to the minimal EGF-like domain (HRG- β 1 177-228) was conducted using monovalent phage display. For these variants, residue numbers also are expressed, in parentheses, in terms of the position of the residue in the minimal EGF-like domain (i.e., HRG- β 1 EGF 1-52).

Variants of HRG- β 1 EGF were prepared and selected for binding to HER-3-Ig using monovalent phage display, according to the method of Bass *et al.*, *Proteins* 8:309-314 (1990). As discussed in detail below, an HRG- β 1 EGF phagemid vector was prepared, in which HRG- β 1 EGF was fused to a C-terminal fragment of the M13 coat protein pIII. Kunkel mutagenesis was performed to introduce stop codons into this vector at sites selected for randomization. This step ensures that the starting vector is incapable of expressing the wild-type polypeptide. Stretches of four to six residues per library were randomized in a linear fashion, except for the six cysteines, Phe189 (HRG- β 1 EGF Phe13) and the two most C-terminal residues. Phe189 was not altered because this residue is conserved as an aromatic residue in EGF and TGF- α and forms a stacking interaction with Tyr208 (HRG- β 1 EGF Tyr32) Jacobsen *et al.*, *Biochemistry* 35:3402-

17 (1996). HRG- β 1 EGF was thus covered in eight libraries, designated A-E, G, H and I.

Library E, covering HRG- β 1 202-209 (HRG- β 1 EGF 26-33), contained a three-residue deletion. The deleted region corresponds to a disordered turn between the second and third β -sheet of HRG- β 1 EGF, and the equivalent amino acids are absent in EGF and TGF- α . An HRG- β 1 EGF control variant in which
 5 HRG- β 1 202-204 (HRG- β 1 EGF 26-28) of HRG8 are deleted (HRG63) bound HER-3-Ig with an affinity similar to that of wild-type.

An additional library (F) was created to randomize a surface patch composed of side chains from the first and second β -sheets, which included HRG- β 1 178, 180, 198, and 200 (HRG- β 1 EGF 2, 4, 22, and 24).

10 The selected sites in the starting vectors were randomized by Kunkel mutagenesis to produce HRG- β 1 EGF libraries. Phage displaying mutated HRG- β 1 EGFs were produced from the libraries under conditions such that, statistically, each phage particle displayed no more than one copy of the mutated HRG- β 1 EGF. See Bass *et al.*, *supra*. These phage were then selected for binding to (sorted against) HER-3-Ig immobilized on an ELISA plate. Bound phage were eluted and used to reinfect host cells, which were used
 15 to produce new phage for another round of sorting. This process was repeated six to seven times for each library. Twelve clones from the phage selected from each library were then sequenced.

Table 2: Library A Variants

		Position in HRG- β 1				
		177	178	179	180	181
20	Wild-type	S	H	L	V	K
	1	W	R	.	.	P
	2	W	S	.	Q	P
	3, 5, 10	W	E	.	.	P
	4	W	S	.	.	.
25	6	W	S	.	I	P
	7	W	R	.	.	A
	8	W	A	.	.	P
	9	W	S	.	Q	.
	11	W	E	.	.	A
30	12	W	S	.	E	P

Table 3: Library B Variants

		Position in HRG- β 1					
		183	184	185	186	187	188
35	Wild-type	A	E	K	E	K	T
	1*	G	V	G	R	D	G
	2*	G	G	E	R	E	G
	3	G	.	E	R	E	G
	4*, 5*	G	W	D	R	E	G
40	6*	G	V	Q	R	E	G
	7	G	.	E	R	A	G
	8	G	K	E	R	E	G
	9*	T	N	S	R	E	G
	10*	D	K	S	R	E	G
45	11*	G	.	D	R	.	Q
	12	G	R	E	R	E	G

*Variant also contained Met226Ile.

Table 4: Library C Variants

		Position in HRG- β 1				
Variant No.		191	192	193	194	195
Wild-type		V	N	G	G	E
1, 2, 4, 5, 7-12	
3		V
6		Q

Table 5: Library D Variants

		Position in HRG- β 1				
Variant No.		197	198	199	200	201
Wild-type		F	M	V	K	D
1*, 2*, 8*, 12*		Y	K	.	R	I
3		.	R	.	.	T
4, 5, 7, 9		Y	R	.	.	T
6		Y	.	I	.	Y
10		Y	.	.	.	T
11		M	R	.	R	T

*Variant also contained Met226Ile.

Table 6: Library E Variants

		Position in HRG- β 1				
Variant No.		205	206	207	208	209
Wild-type		P	S	R	Y	L
1		T	P	Y	L	M
2, 4		Y	G	Y	L	M
3*		Y	R	Y	R	M
5, 12		T	H	Y	R	G
6		T	H	Y	R	M
7*		Y	K	Y	R	M
8, 9		T	K	Y	R	G
10		Y	K	Y	R	.

*Variant also contained Met226Ile.

Table 7: Library G Variants

		Position in HRG- β 1					
Variant No.		211	212	213	214	215	216
Wild-type		K	C	P	N	E	F
1, 5, 6, 10, 12		R	.	S	L	.	.
2		R	.	S	E	.	.
3		.	.	.	K	.	M
4		R	.	T	V	.	Y
7, 8		R	.	T	V	.	Y
9		.	.	N	S	.	.
11		R	.	K	K	.	.

Example 2 - HER2/HER3 expression in embryonic rat lung

Rat lungs were microdissected from rat embryos on embryologic day (E) 16, 18, 20, and post-natal (P) days 7, 14, and adult. Isolated lung tissue was homogenized in a standard protease inhibitor buffer, and equal protein amounts subjected to SDS-PAGE (4-20%), blotted to nitrocellulose and identified with specific antibodies (HER2, HER3, HER4-Santa Cruz Biological, San Jose, CA. and HRG, 3G11, Genentech, Inc.) using chemiluminescent techniques.

Analysis of the blot indicates that HER2 is expressed at high levels throughout development in utero, appears to peak on E18 and declines after birth to lower adult levels. HER3 expression peaks at E18,

and then declines after birth to lower adult levels. HER4 is not identified at any time during lung development. A proform of HRG may be present (75 kDa protein) on E16, peaking on E18 and then declining to low levels in the adult. These data suggest that the HER/HRG system is developmentally modulated during lung development and the active receptor may be a HER2/HER3 heterodimer. The time period during which the receptors peak in expression (E18) represents the pseudoglandular stage of rat lung development (days 13-18), bordering on the canalicular stage (days 19-20). During the pseudoglandular stage, pulmonary epithelial cell proliferation is higher than at any other time. During the canalicular stage, differentiation begins with the appearance of type I pneumocyte and type II (surfactant producing) pneumocytes. This indicates a role for HRG/HER interaction in either or both processes.

10 **Example 3 - HER2/HER3 expression in fetal human lung**

Fetal human lung was obtained from mid trimester embryos (17-22 weeks). Lung tissue was cultured in serum free Weymouth's media at an air fluid interface at 37°C in a humidified 5% CO₂ atmosphere. Tissue was harvested from the culture on days 0 (day tissue was received) and after 1-4 days (D) in culture (D1-D4), homogenized in a standard protease inhibitor buffer, equal protein amounts subjected to SDS-PAGE (4-20%), blotted to nitrocellulose and identified with specific antibodies.

HER2 is expressed on D0 and increases in expression level during *in vitro* culture. HER3 is present at low to undetectable levels at D0 and increases in expression level during *in vitro* culture. HER4 was not identified at any time during lung development. HRG was not identified on D0, however, it was identified as a 75,000 Da protein on D1-D2 and continued to rise in expression level throughout time in culture. This human explant model recapitulates part of the normal lung developmental program over the 5 days in culture. Development occurs rapidly with both epithelial cell proliferation, and differentiation occurring, along with air space formation. These data indicate that the HER/HRG system is also modulated during *in vitro* human lung development, and the active receptor may be a HER2/HER3 heterodimer. The third trimester represents late pseudoglandular (days 42-112) and canalicular stage (days 112-196) in human lung development. As in the rat, during the pseudoglandular stage epithelial cell proliferation and formation of the prospective airways occurs. During the canalicular stage differentiation of the epithelium occurs.

25 **Example 4 - Expression of HER2 and HER3 in human lung**

HER2 and HER3 are expressed exclusively in the pulmonary epithelium during lung development. Mid-trimester human lung was cultured *in vitro* as outlined above. Tissue was harvested daily, snap frozen, and 5 micron sections cut. The sections were mounted on glass slides, and immunohistochemistry performed using standard ABC procedures. HER2, HER3, and HER4 were identified using specific antibodies as described above.

As a control, lung tissue was stained at D0 and D5 with an irrelevant antibody and no staining was detected. At D0, the lung is relatively unformed. The majority of the tissue is mesenchymal cells. Early air spaces are being developed. On D5, air spaces are clearly identifiable, with thinning of the mesenchymal tissue and proliferation of the lung epithelium required to cover the enlarging air spaces.

Using HER2 staining at D0 and D5, it was established that HER 2 is uniformly present on D0 lung tissue throughout the lung epithelial tissue. No expression was present in the mesenchymal tissue. By D5, HER2 remains uniform and restricted to the pulmonary epithelium.

Using HER3 staining control at D0 and D5, HER3 was identifiable in D0 lung tissue. The staining was relatively less than HER2, and was not homogenous, suggesting that there are specific epithelial areas expressing the HER3 receptor. By D5, expression had become more homogenous throughout the lung epithelium, but clearly not uniform. Expression remained epithelium specific.

40 **Example 5 - Preparation of rHRG β 1₁₇₇₋₂₄₄**

The EGF-like domain fragment HRG- β 1 177-244 was amplified from vector pHL89 (which is described in Holmes et al., *Science* 256:1205-1210 (1992)) by PCR with primers having NsiI/XbaI-

containing overhangs. The fragment was inserted into phagemid display vector pam-g3 by restriction digestion at the same sites to generate construct pHRG2-g3 (177-244). pam-g3 was a derivative of phGHam-g3, which was designed for phage display of human growth hormone (hGH) and was described in Lowman et al., *Biochemistry* 30:10832-10838 (1991). pam-g3 was produced by removing the hGH gene present in phGHam-g3 and replacing this gene with a stuffer fragment, which provides space for cleavage at the restriction sites used for cloning. The HRG- β 1 fragment was attached to residue 247 of pIII.

The HRG- β 1 EGF-like domain expressed from the above-described construct is designated by removing the "p" and the "-g3" that appear in the name of the construct. Thus, the HRG- β 1 EGF-like domain expressed from the pHRG2-g3 construct is designated "HRG2."

The domain was displayed monovalently on phage as a pIII fusion protein, as described by Bass et al., *Proteins* 8:309-314 (1990).

Similarly, variants HRG- β 1₁₄₇₋₂₂₇, HRG- β 1₁₄₇₋₂₄₄, and HRG- β 1₁₇₇₋₂₂₇ were prepared and expressed as described above.

Example 6 - rHRG β 1₁₇₇₋₂₄₄ causes accelerated lung development

Exogenous rHRG β 1₁₇₇₋₂₄₄ caused accelerated lung development *in vitro*. To determine if the expressed HER2/HER3 receptors were functional and the role of HRG stimulation during lung development *in vitro*, rHRG β 1₁₇₇₋₂₄₄ was added to the *in vitro* culture at 10 nM. Tissue was harvested at D5, snap frozen, and 5 micron sections were cut for analysis.

The morphology, in comparison to the untreated control specimens was grossly different. There was marked proliferation of the epithelium. Air spaces which are typically lined with a single cell layer now had a 2-3 cell thickness. The changes were dose dependent with more epithelial cell response with higher concentrations. HER2 and HER3 were still identifiable in the epithelium only.

Example 7 - Human lung differentiation

Differentiation of human lung epithelial cells occurs after HRG treatment. Differentiation was measured by Surfactant Protein A (SPA) production. All sections were stained for SPA. Human lung explant stained for SPA with stain localizing in epithelial cells of the prealveolar ducts. Human lung explant exposed to 10 nM HRG showed an effect on SPA production. As a differentiation control, a lung explant was exposed to 1mM dibutyryl cAMP. A negative control was also run.

WE CLAIM:

1. A method of inducing epithelial cell growth and/or proliferation, comprising contacting a normal epithelial cell which expresses HER2, HER3 and/or HER4 receptors with an effective amount of an isolated ligand which activates HER2, HER3, HER4 receptors or a combination thereof.
2. The method of Claim 1, wherein the activating ligand is a heregulin (HRG) polypeptide, HRG variant, HRG agonist antibody or fragment thereof capable of binding to the HER2, HER3 and/or HER4 receptor.
3. The method of Claim 2, wherein the activating ligand is human HRG or a fragment thereof.
4. The method of Claim 2, wherein the activating ligand is selected from the group consisting of HRG- α , - β 1, - β 2, - β 2-like, and - β 3 and fragments thereof.
5. The method of Claim 2, wherein the activating ligand is γ -HRG or a fragment thereof.
6. The method of Claim 2, wherein the activating ligand is recombinant human HRG or a fragment thereof.
7. The method of Claim 2, wherein the activating ligand is sensory and motor neuron derived factor (SMDF) or a fragment thereof.
8. The method of Claim 1, wherein the activating ligand is administered at a daily dose of about 1 μ g/kg to 100 mg/kg.
9. The method of Claim 2, wherein the activating ligand is an agonist antibody.
10. The method of Claim 1, wherein the contacting is by administration to a patient in need thereof.
11. The method of Claim 6, wherein the HRG is rHRG- β 1-177-244.
12. The method of Claim 1, wherein the epithelial cell is a lung cell.
13. The method of Claim 1 wherein the epithelial cell expresses HER2/HER3, HER2/HER4, HER3/HER4, HER3 or HER4.
14. A method of increasing lung surfactant protein A, comprising administering to a patient in need thereof an effective amount of an isolated HER2, HER3 and/or HER4 activating ligand.
15. The method of Claim 14, wherein the activating ligand is a heregulin (HRG) polypeptide, HRG variant, HRG agonist antibody or fragment thereof capable of binding to the HER2, HER3 and/or HER4 receptor.
16. A method of treating chronic obstructive pulmonary disease, comprising administering to a patient in need thereof an effective amount of an isolated HER2, HER3 and/or HER4 activating ligand.

17. The method of Claim 16, wherein the activating ligand is a heregulin (HRG) polypeptide, HRG variant, HRG agonist antibody or fragment thereof capable of binding to the HER2, HER3 and/or HER4 receptor.

18. A method of treating respiratory distress or emphysema, comprising administering to a patient in need thereof an effective amount of an isolated HER2, HER3 and/or HER4 activating ligand.

19. The method of Claim 18, wherein the activating ligand is a heregulin (HRG) polypeptide, HRG variant, HRG agonist antibody or fragment thereof capable of binding to the HER2, HER3 and/or HER4 receptor.

20. A method, comprising the steps of:

- (a) obtaining a normal epithelial cell sample from a mammal;
- (b) contacting the sample with a ligand which activates HER2, HER3, HER4 or a combination thereof to induce growth and/or proliferation of epithelial cells in the sample and to obtain an expanded sample; and
- (c) re-introducing the expanded sample into the mammal.

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GG	GCG	CGA	GCG	CCT	CAG	CGC	GGC	CGC	TCG	CTC	TCC	CCC	38
	Ala	Arg	Ala	Pro	Gln	Arg	Gly	Arg	Ser	Leu	Ser	Pro	
	1				5					10			
TCG	AGG	GAC	AAA	CTT	TTC	CCA	AAC	CCG	ATC	CGA	GCC	CTT	77
Ser	Arg	Asp	Lys	Leu	Phe	Pro	Asn	Pro	Ile	Arg	Ala	Leu	
	15						20					25	
GGA	CCA	AAC	TCG	CCT	GCG	CCG	AGA	GCC	GTC	CGC	GTA	GAG	116
Gly	Pro	Asn	Ser	Pro	Ala	Pro	Arg	Ala	Val	Arg	Val	Glu	
				30					35				
CGC	TCC	GTC	TCC	GGC	GAG	ATG	TCC	GAG	CGC	AAA	GAA	GGC	155
Arg	Ser	Val	Ser	Gly	Glu	Met	Ser	Glu	Arg	Lys	Glu	Gly	
	40					45					50		
AGA	GGC	AAA	GGG	AAG	GGC	AAG	AAG	AAG	GAG	CGA	GGC	TCC	194
Arg	Gly	Lys	Gly	Lys	Gly	Lys	Lys	Lys	Glu	Arg	Gly	Ser	
			55					60					
GGC	AAG	AAG	CCG	GAG	TCC	GCG	GCG	GGC	AGC	CAG	AGC	CCA	233
Gly	Lys	Lys	Pro	Glu	Ser	Ala	Ala	Gly	Ser	Gln	Ser	Pro	
	65				70					75			
GCC	TTG	CCT	CCC	CGA	TTG	AAA	GAG	ATG	AAA	AGC	CAG	GAA	272
Ala	Leu	Pro	Pro	Arg	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu	
		80					85					90	
TCG	GCT	GCA	GGT	TCC	AAA	CTA	GTC	CTT	CGG	TGT	GAA	ACC	311
Ser	Ala	Ala	Gly	Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	
				95					100				
AGT	TCT	GAA	TAC	TCC	TCT	CTC	AGA	TTC	AAG	TGG	TTC	AAG	350
Ser	Ser	Glu	Tyr	Ser	Ser	Leu	Arg	Phe	Lys	Trp	Phe	Lys	
	105					110					115		
AAT	GGG	AAT	GAA	TTG	AAT	CGA	AAA	AAC	AAA	CCA	CAA	AAT	389
Asn	Gly	Asn	Glu	Leu	Asn	Arg	Lys	Asn	Lys	Pro	Gln	Asn	
			120					125					
ATC	AAG	ATA	CAA	AAA	AAG	CCA	GGG	AAG	TCA	GAA	CTT	CGC	428
Ile	Lys	Ile	Gln	Lys	Lys	Pro	Gly	Lys	Ser	Glu	Leu	Arg	
	130				135					140			
ATT	AAC	AAA	GCA	TCA	CTG	GCT	GAT	TCT	GGA	GAG	TAT	ATG	467
Ile	Asn	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr	Met	
		145				150						155	
TGC	AAA	GTG	ATC	AGC	AAA	TTA	GGA	AAT	GAC	AGT	GCC	TCT	506
Cys	Lys	Val	Ile	Ser	Lys	Leu	Gly	Asn	Asp	Ser	Ala	Ser	
				160					165				

FIG. 1A

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GCC	AAT	ATC	ACC	ATC	GTG	GAA	TCA	AAC	GAG	ATC	ATC	ACT	545
Ala	Asn	Ile	Thr	Ile	Val	Glu	Ser	Asn	Glu	Ile	Ile	Thr	
170						175						180	
GGT	ATG	CCA	GCC	TCA	ACT	GAA	GGA	GCA	TAT	GTG	TCT	TCA	584
Gly	Met	Pro	Ala	Ser	Thr	Glu	Gly	Ala	Tyr	Val	Ser	Ser	
			185					190					
GAG	TCT	CCC	ATT	AGA	ATA	TCA	GTA	TCC	ACA	GAA	GGA	GCA	623
Glu	Ser	Pro	Ile	Arg	Ile	Ser	Val	Ser	Thr	Glu	Gly	Ala	
195					200					205			
AAT	ACT	TCT	TCA	TCT	ACA	TCT	ACA	TCC	ACC	ACT	GGG	ACA	662
Asn	Thr	Ser	Ser	Ser	Thr	Ser	Thr	Ser	Thr	Thr	Gly	Thr	
		210					215					220	
AGC	CAT	CTT	GTA	AAA	TGT	GCG	GAG	AAG	GAG	AAA	ACT	TTC	701
Ser	His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	
				225					230				
TGT	GTG	AAT	GGA	GGG	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	740
Cys	Val	Asn	Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	
235					240						245		
TCA	AAC	CCC	TCG	AGA	TAC	TTG	TGC	AAG	TGC	CAA	CCT	GGA	779
Ser	Asn	Pro	Ser	Arg	Tyr	Leu	Cys	Lys	Cys	Gln	Pro	Gly	
			250					255					
TTC	ACT	GGA	GCA	AGA	TGT	ACT	GAG	AAT	GTG	CCC	ATG	AAA	818
Phe	Thr	Gly	Ala	Arg	Cys	Thr	Glu	Asn	Val	Pro	Met	Lys	
260					265					270			
GTC	CAA	AAC	CAA	GAA	AAG	GCG	GAG	GAG	CTG	TAC	CAG	AAG	857
Val	Gln	Asn	Gln	Glu	Lys	Ala	Glu	Glu	Leu	Tyr	Gln	Lys	
		275					280					285	
AGA	GTG	CTG	ACC	ATA	ACC	GGC	ATC	TGC	ATC	GCC	CTC	CTT	896
Arg	Val	Leu	Thr	Ile	Thr	Gly	Ile	Cys	Ile	Ala	Leu	Leu	
				290					295				
GTG	GTC	GGC	ATC	ATG	TGT	GTG	GTG	GCC	TAC	TGC	AAA	ACC	935
Val	Val	Gly	Ile	Met	Cys	Val	Val	Ala	Tyr	Cys	Lys	Thr	
300						305					310		
AAG	AAA	CAG	CGG	AAA	AAG	CTG	CAT	GAC	CGT	CTT	CGG	CAG	974
Lys	Lys	Gln	Arg	Lys	Lys	Leu	His	Asp	Arg	Leu	Arg	Gln	
			315					320					
AGC	CTT	CGG	TCT	GAA	CGA	AAC	AAT	ATG	ATG	AAC	ATT	GCC	1013
Ser	Leu	Arg	Ser	Glu	Arg	Asn	Asn	Met	Met	Asn	Ile	Ala	
325					330					335			

FIG. 1B

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AAT	GGG	CCT	CAC	CAT	CCT	AAC	CCA	CCC	CCC	GAG	AAT	GTC	1052
Asn	Gly	Pro	His	His	Pro	Asn	Pro	Pro	Pro	Glu	Asn	Val	
		340					345					350	
CAG	CTG	GTG	AAT	CAA	TAC	GTA	TCT	AAA	AAC	GTC	ATC	TCC	1091
Gln	Leu	Val	Asn	Gln	Tyr	Val	Ser	Lys	Asn	Val	Ile	Ser	
				355					360				
AGT	GAG	CAT	ATT	GTT	GAG	AGA	GAA	GCA	GAG	ACA	TCC	TTT	1130
Ser	Glu	His	Ile	Val	Glu	Arg	Glu	Ala	Glu	Thr	Ser	Phe	
	365					370					375		
TCC	ACC	AGT	CAC	TAT	ACT	TCC	ACA	GCC	CAT	CAC	TCC	ACT	1169
Ser	Thr	Ser	His	Tyr	Thr	Ser	Thr	Ala	His	His	Ser	Thr	
			380					385					
ACT	GTC	ACC	CAG	ACT	CCT	AGC	CAC	AGC	TGG	AGC	AAC	GGA	1208
Thr	Val	Thr	Gln	Thr	Pro	Ser	His	Ser	Trp	Ser	Asn	Gly	
	390				395					400			
CAC	ACT	GAA	AGC	ATC	CTT	TCC	GAA	AGC	CAC	TCT	GTA	ATC	1247
His	Thr	Glu	Ser	Ile	Leu	Ser	Glu	Ser	His	Ser	Val	Ile	
		405					410					415	
GTG	ATG	TCA	TCC	GTA	GAA	AAC	AGT	AGG	CAC	AGC	AGC	CCA	1286
Val	Met	Ser	Ser	Val	Glu	Asn	Ser	Arg	His	Ser	Ser	Pro	
				420					425				
ACT	GGG	GGC	CCA	AGA	GGA	CGT	CTT	AAT	GGC	ACA	GGA	GGC	1325
Thr	Gly	Gly	Pro	Arg	Gly	Arg	Leu	Asn	Gly	Thr	Gly	Gly	
	430					435					440		
CCT	CGT	GAA	TGT	AAC	AGC	TTC	CTC	AGG	CAT	GCC	AGA	GAA	1364
Pro	Arg	Glu	Cys	Asn	Ser	Phe	Leu	Arg	His	Ala	Arg	Glu	
			445					450					
ACC	CCT	GAT	TCC	TAC	CGA	GAC	TCT	CCT	CAT	AGT	GAA	AGG	1403
Thr	Pro	Asp	Ser	Tyr	Arg	Asp	Ser	Pro	His	Ser	Glu	Arg	
	455				460					465			
TAT	GTG	TCA	GCC	ATG	ACC	ACC	CCG	GCT	CGT	ATG	TCA	CCT	1442
Tyr	Val	Ser	Ala	Met	Thr	Thr	Pro	Ala	Arg	Met	Ser	Pro	
		470					475					480	
GTA	GAT	TTC	CAC	ACG	CCA	AGC	TCC	CCC	AAA	TCG	CCC	CCT	1481
Val	Asp	Phe	His	Thr	Pro	Ser	Ser	Pro	Lys	Ser	Pro	Pro	
				485					490				
TCG	GAA	ATG	TCT	CCA	CCC	GTG	TCC	AGC	ATG	ACG	GTG	TCC	1520
Ser	Glu	Met	Ser	Pro	Pro	Val	Ser	Ser	Met	Thr	Val	Ser	
	495					500					505		

FIG. 1C

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ATG	CCT	TCC	ATG	GCG	GTC	AGC	CCC	TTC	ATG	GAA	GAA	GAG	1559
Met	Pro	Ser	Met	Ala	Val	Ser	Pro	Phe	Met	Glu	Glu	Glu	
			510					515					
AGA	CCT	CTA	CTT	CTC	GTG	ACA	CCA	CCA	AGG	CTG	CGG	GAG	1598
Arg	Pro	Leu	Leu	Leu	Val	Thr	Pro	Pro	Arg	Leu	Arg	Glu	
520					525					530			
AAG	AAG	TTT	GAC	CAT	CAC	CCT	CAG	CAG	TTC	AGC	TCC	TTC	1637
Lys	Lys	Phe	Asp	His	His	Pro	Gln	Gln	Phe	Ser	Ser	Phe	
		535					540					545	
CAC	CAC	AAC	CCC	GCG	CAT	GAC	AGT	AAC	AGC	CTC	CCT	GCT	1676
His	His	Asn	Pro	Ala	His	Asp	Ser	Asn	Ser	Leu	Pro	Ala	
				550					555				
AGC	CCC	TTG	AGG	ATA	GTG	GAG	GAT	GAG	GAG	TAT	GAA	ACG	1715
Ser	Pro	Leu	Arg	Ile	Val	Glu	Asp	Glu	Glu	Tyr	Glu	Thr	
	560					565					570		
ACC	CAA	GAG	TAC	GAG	CCA	GCC	CAA	GAG	CCT	GTT	AAG	AAA	1754
Thr	Gln	Glu	Tyr	Glu	Pro	Ala	Gln	Glu	Pro	Val	Lys	Lys	
			575						580				
CTC	GCC	AAT	AGC	CGG	CGG	GCC	AAA	AGA	ACC	AAG	CCC	AAT	1793
Leu	Ala	Asn	Ser	Arg	Arg	Ala	Lys	Arg	Thr	Lys	Pro	Asn	
585					590					595			
GGC	CAC	ATT	GCT	AAC	AGA	TTG	GAA	GTG	GAC	AGC	AAC	ACA	1832
Gly	His	Ile	Ala	Asn	Arg	Leu	Glu	Val	Asp	Ser	Asn	Thr	
		600					605					610	
AGC	TCC	CAG	AGC	AGT	AAC	TCA	GAG	AGT	GAA	ACA	GAA	GAT	1871
Ser	Ser	Gln	Ser	Ser	Asn	Ser	Glu	Ser	Glu	Thr	Glu	Asp	
				615					620				
GAA	AGA	GTA	GGT	GAA	GAT	ACG	CCT	TTC	CTG	GGC	ATA	CAG	1910
Glu	Arg	Val	Gly	Glu	Asp	Thr	Pro	Phe	Leu	Gly	Ile	Gln	
	625					630					635		
AAC	CCC	CTG	GCA	GCC	AGT	CTT	GAG	GCA	ACA	CCT	GCC	TTC	1949
Asn	Pro	Leu	Ala	Ala	Ser	Leu	Glu	Ala	Thr	Pro	Ala	Phe	
			640					645					
CGC	CTG	GCT	GAC	AGC	AGG	ACT	AAC	CCA	GCA	GGC	CGC	TTC	1988
Arg	Leu	Ala	Asp	Ser	Arg	Thr	Asn	Pro	Ala	Gly	Arg	Phe	
650					655					660			
TCG	ACA	CAG	GAA	GAA	ATC	CAG	G						2010
Ser	Thr	Gln	Glu	Glu	Ile	Gln							
		665				669							

FIG. 1D

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GG	GAC	AAA	CTT	TTC	CCA	AAC	CCG	ATC	CGA	GCC	CTT	GGA	38
	Asp	Lys	Leu	Phe	Pro	Asn	Pro	Ile	Arg	Ala	Leu	Gly	
	1				5					10			
CCA	AAC	TCG	CCT	GCG	CCG	AGA	GCC	GTC	CGC	GTA	GAG	CGC	77
Pro	Asn	Ser	Pro	Ala	Pro	Arg	Ala	Val	Arg	Val	Glu	Arg	
		15					20					25	
TCC	GTC	TCC	GGC	GAG	ATG	TCC	GAG	CGC	AAA	GAA	GGC	AGA	116
Ser	Val	Ser	Gly	Glu	Met	Ser	Glu	Arg	Lys	Glu	Gly	Arg	
			30						35				
GGC	AAA	GGG	AAG	GGC	AAG	AAG	AAG	GAG	CGA	GGC	TCC	GGC	155
Gly	Lys	Gly	Lys	Gly	Lys	Lys	Lys	Glu	Arg	Gly	Ser	Gly	
	40					45					50		
AAG	AAG	CCG	GAG	TCC	GCG	GCG	GGC	AGC	CAG	AGC	CCA	GCC	194
Lys	Lys	Pro	Glu	Ser	Ala	Ala	Gly	Ser	Gln	Ser	Pro	Ala	
			55					60					
TTG	CCT	CCC	CAA	TTG	AAA	GAG	ATG	AAA	AGC	CAG	GAA	TCG	233
Leu	Pro	Pro	Gln	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu	Ser	
	65				70					75			
GCT	GCA	GGT	TCC	AAA	CTA	GTC	CTT	CGG	TGT	GAA	ACC	AGT	272
Ala	Ala	Gly	Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	
		80					85					90	
TCT	GAA	TAC	TCC	TCT	CTC	AGA	TTC	AAG	TGG	TTC	AAG	AAT	311
Ser	Glu	Tyr	Ser	Ser	Leu	Arg	Phe	Lys	Trp	Phe	Lys	Asn	
				95					100				
GGG	AAT	GAA	TTG	AAT	CGA	AAA	AAC	AAA	CCA	CAA	AAT	ATC	350
Gly	Asn	Glu	Leu	Asn	Arg	Lys	Asn	Lys	Pro	Gln	Asn	Ile	
	105					110					115		
AAG	ATA	CAA	AAA	AAG	CCA	GGG	AAG	TCA	GAA	CTT	CGC	ATT	389
Lys	Ile	Gln	Lys	Lys	Pro	Gly	Lys	Ser	Glu	Leu	Arg	Ile	
			120					125					
AAC	AAA	GCA	TCA	CTG	GCT	GAT	TCT	GGA	GAG	TAT	ATG	TGC	428
Asn	Lys	Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr	Met	Cys	
	130				135					140			
AAA	GTG	ATC	AGC	AAA	TTA	GGA	AAT	GAC	AGT	GCC	TCT	GCC	467
Lys	Val	Ile	Ser	Lys	Leu	Gly	Asn	Asp	Ser	Ala	Ser	Ala	
		145					150					155	
AAT	ATC	ACC	ATC	GTG	GAA	TCA	AAC	GAG	ATC	ATC	ACT	GGT	506
Asn	Ile	Thr	Ile	Val	Glu	Ser	Asn	Glu	Ile	Ile	Thr	Gly	
				160					165				

FIG. 2A

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ATG	CCA	GCC	TCA	ACT	GAA	GGA	GCA	TAT	GTG	TCT	TCA	GAG	545
Met	Pro	Ala	Ser	Thr	Glu	Gly	Ala	Tyr	Val	Ser	Ser	Glu	
	170					175					180		
TCT	CCC	ATT	AGA	ATA	TCA	GTA	TCC	ACA	GAA	GGA	GCA	AAT	584
Ser	Pro	Ile	Arg	Ile	Ser	Val	Ser	Thr	Glu	Gly	Ala	Asn	
			185				190						
ACT	TCT	TCA	TCT	ACA	TCT	ACA	TCC	ACC	ACT	GGG	ACA	AGC	623
Thr	Ser	Ser	Ser	Thr	Ser	Thr	Ser	Thr	Thr	Gly	Thr	Ser	
195					200					205			
CAT	CTT	GTA	AAA	TGT	GCG	GAG	AAG	GAG	AAA	ACT	TTC	TGT	662
His	Leu	Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	
		210					215					220	
GTG	AAT	GGA	GGG	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	TCA	701
Val	Asn	Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	
				225					230				
AAC	CCC	TCG	AGA	TAC	TTG	TGC	AAG	TGC	CCA	AAT	GAG	TTT	740
Asn	Pro	Ser	Arg	Tyr	Leu	Cys	Lys	Cys	Pro	Asn	Glu	Phe	
	235					240					245		
ACT	GGT	GAT	CGC	TGC	CAA	AAC	TAC	GTA	ATG	GCC	AGC	TTC	779
Thr	Gly	Asp	Arg	Cys	Gln	Asn	Tyr	Val	Met	Ala	Ser	Phe	
			250					255					
TAC	AAG	CAT	CTT	GGG	ATT	GAA	TTT	ATG	GAG	GCG	GAG	GAG	818
Tyr	Lys	His	Leu	Gly	Ile	Glu	Phe	Met	Glu	Ala	Glu	Glu	
260					265					270			
CTG	TAC	CAG	AAG	AGA	GTG	CTG	ACC	ATA	ACC	GGC	ATC	TGC	857
Leu	Tyr	Gln	Lys	Arg	Val	Leu	Thr	Ile	Thr	Gly	Ile	Cys	
		275					280					285	
ATC	GCC	CTC	CTT	GTG	GTC	GGC	ATC	ATG	TGT	GTG	GTG	GCC	896
Ile	Ala	Leu	Leu	Val	Val	Gly	Ile	Met	Cys	Val	Val	Ala	
				290					295				
TAC	TGC	AAA	ACC	AAG	AAA	CAG	CGG	AAA	AAG	CTG	CAT	GAC	935
Tyr	Cys	Lys	Thr	Lys	Lys	Gln	Arg	Lys	Lys	Leu	His	Asp	
	300					305					310		
CGT	CTT	CGG	CAG	AGC	CTT	CGG	TCT	GAA	CGA	AAC	AAT	ATG	974
Arg	Leu	Arg	Gln	Ser	Leu	Arg	Ser	Glu	Arg	Asn	Asn	Met	
			315					320					
ATG	AAC	ATT	GCC	AAT	GGG	CCT	CAC	CAT	CCT	AAC	CCA	CCC	1013
Met	Asn	Ile	Ala	Asn	Gly	Pro	His	His	Pro	Asn	Pro	Pro	
325					330					335			

FIG. 2B

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CCC	GAG	AAT	GTC	CAG	CTG	GTG	AAT	CAA	TAC	GTA	TCT	AAA	1052
Pro	Glu	Asn	Val	Gln	Leu	Val	Asn	Gln	Tyr	Val	Ser	Lys	
		340					345					350	
AAC	GTC	ATC	TCC	AGT	GAG	CAT	ATT	GTT	GAG	AGA	GAA	GCA	1091
Asn	Val	Ile	Ser	Ser	Glu	His	Ile	Val	Glu	Arg	Glu	Ala	
				355					360				
GAG	ACA	TCC	TTT	TCC	ACC	AGT	CAC	TAT	ACT	TCC	ACA	GCC	1130
Glu	Thr	Ser	Phe	Ser	Thr	Ser	His	Tyr	Thr	Ser	Thr	Ala	
	365					370					375		
CAT	CAC	TCC	ACT	ACT	GTC	ACC	CAG	ACT	CCT	AGC	CAC	AGC	1169
His	His	Ser	Thr	Thr	Val	Thr	Gln	Thr	Pro	Ser	His	Ser	
			380					385					
TGG	AGC	AAC	GGA	CAC	ACT	GAA	AGC	ATC	CTT	TCC	GAA	AGC	1208
Trp	Ser	Asn	Gly	His	Thr	Glu	Ser	Ile	Leu	Ser	Glu	Ser	
390					395				400				
CAC	TCT	GTA	ATC	GTG	ATG	TCA	TCC	GTA	GAA	AAC	AGT	AGG	1247
His	Ser	Val	Ile	Val	Met	Ser	Ser	Val	Glu	Asn	Ser	Arg	
		405					410					415	
CAC	AGC	AGC	CCA	ACT	GGG	GGC	CCA	AGA	GGA	CGT	CTT	AAT	1286
His	Ser	Ser	Pro	Thr	Gly	Gly	Pro	Arg	Gly	Arg	Leu	Asn	
				420					425				
GGC	ACA	GGA	GGC	CCT	CGT	GAA	TGT	AAC	AGC	TTC	CTC	AGG	1325
Gly	Thr	Gly	Gly	Pro	Arg	Glu	Cys	Asn	Ser	Phe	Leu	Arg	
	430					435					440		
CAT	GCC	AGA	GAA	ACC	CCT	GAT	TCC	TAC	CGA	GAC	TCT	CCT	1364
His	Ala	Arg	Glu	Thr	Pro	Asp	Ser	Tyr	Arg	Asp	Ser	Pro	
			445					450					
CAT	AGT	GAA	AGG	TAT	GTG	TCA	GCC	ATG	ACC	ACC	CCG	GCT	1403
His	Ser	Glu	Arg	Tyr	Val	Ser	Ala	Met	Thr	Thr	Pro	Ala	
455					460					465			
CGT	ATG	TCA	CCT	GTA	GAT	TTC	CAC	ACG	CCA	AGC	TCC	CCC	1442
Arg	Met	Ser	Pro	Val	Asp	Phe	His	Thr	Pro	Ser	Ser	Pro	
		470					475					480	
AAA	TCG	CCC	CCT	TCG	GAA	ATG	TCT	CCA	CCC	GTG	TCC	AGC	1481
Lys	Ser	Pro	Pro	Ser	Glu	Met	Ser	Pro	Pro	Val	Ser	Ser	
				485					490				
ATG	ACG	GTG	TCC	ATG	CCT	TCC	ATG	GCG	GTC	AGC	CCC	TTC	1520
Met	Thr	Val	Ser	Met	Pro	Ser	Met	Ala	Val	Ser	Pro	Phe	
	495					500					505		

FIG._2C

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ATG	GAA	GAA	GAG	AGA	CCT	CTA	CTT	CTC	GTG	ACA	CCA	CCA	1559
Met	Glu	Glu	Glu	Arg	Pro	Leu	Leu	Leu	Val	Thr	Pro	Pro	
			510					515					
AGG	CTG	CGG	GAG	AAG	AAG	TTT	GAC	CAT	CAC	CCT	CAG	CAG	1598
Arg	Leu	Arg	Glu	Lys	Lys	Phe	Asp	His	His	Pro	Gln	Gln	
520					525					530			
TTC	AGC	TCC	TTC	CAC	CAC	AAC	CCC	GCG	CAT	GAC	AGT	AAC	1637
Phe	Ser	Ser	Phe	His	His	Asn	Pro	Ala	His	Asp	Ser	Asn	
		535					540					545	
AGC	CTC	CCT	GCT	AGC	CCC	TTG	AGG	ATA	GTG	GAG	GAT	GAG	1676
Ser	Leu	Pro	Ala	Ser	Pro	Leu	Arg	Ile	Val	Glu	Asp	Glu	
			550						555				
GAG	TAT	GAA	ACG	ACC	CAA	GAG	TAC	GAG	CCA	GCC	CAA	GAG	1715
Glu	Tyr	Glu	Thr	Thr	Gln	Glu	Tyr	Glu	Pro	Ala	Gln	Glu	
	560					565					570		
CCT	GTT	AAG	AAA	CTC	GCC	AAT	AGC	CGG	CGG	GCC	AAA	AGA	1754
Pro	Val	Lys	Lys	Leu	Ala	Asn	Ser	Arg	Arg	Ala	Lys	Arg	
			575					580					
ACC	AAG	CCC	AAT	GGC	CAC	ATT	GCT	AAC	AGA	TTG	GAA	GTG	1793
Thr	Lys	Pro	Asn	Gly	His	Ile	Ala	Asn	Arg	Leu	Glu	Val	
585					590					595			
GAC	AGC	AAC	ACA	AGC	TCC	CAG	AGC	AGT	AAC	TCA	GAG	AGT	1832
Asp	Ser	Asn	Thr	Ser	Ser	Gln	Ser	Ser	Asn	Ser	Glu	Ser	
		600					605					610	

FIG._2D

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GAA	ACA	GAA	GAT	GAA	AGA	GTA	GGT	GAA	GAT	ACG	CCT	TTC	1871
Glu	Thr	Glu	Asp	Glu	Arg	Val	Gly	Glu	Asp	Thr	Pro	Phe	
				615						620			
CTG	GGC	ATA	CAG	AAC	CCC	CTG	GCA	GCC	AGT	CTT	GAG	GCA	1910
Leu	Gly	Ile	Gln	Asn	Pro	Leu	Ala	Ala	Ser	Leu	Glu	Ala	
	625					630					635		
ACA	CCT	GCC	TTC	CGC	CTG	GCT	GAC	AGC	AGG	ACT	AAC	CCA	1949
Thr	Pro	Ala	Phe	Arg	Leu	Ala	Asp	Ser	Arg	Thr	Asn	Pro	
			640					645					
GCA	GGC	CGC	TTC	TCG	ACA	CAG	GAA	GAA	ATC	CAG	GCC	AGG	1988
Ala	Gly	Arg	Phe	Ser	Thr	Gln	Glu	Glu	Ile	Gln	Ala	Arg	
	650					655					660		
CTG	TCT	AGT	GTA	ATT	GCT	AAC	CAA	GAC	CCT	ATT	GCT	GTA	TA 2029
Leu	Ser	Ser	Val	Ile	Ala	Asn	Gln	Asp	Pro	Ile	Ala	Val	
		665					670					675	
A AACCTAAATA AACACATAGA TTCACCTGTA AAACCTTTATT													2070
TTATATAATA AAGTATTCCA CCTTAAATTA AACAATTTAT TTTATTTTAG													2120
CAGTTCTGCA AATAGAAAAC AGGAAAAAAA CTTTATATAA TTAAATATAT													2170
GTATGTAAAA ATGAAAAAAA AAAAAAAA													2199

FIG._2E

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GTGGCTGCGG GGCAATTGAA AAAGAGCCGG CGAGGAGTTC CCCGAAACTT 50

GTTGGAAGTC CGGGCTCGCG CGGAGGCCAG GAGCTGAGCG GCGGCGGCTG 100

COGGACGATG GGAGCGTGAG CAGGACGGTG ATAACCTCTC CCCGATCGGG 150

TTGCGAGGGC GCCGGGCAGA GGCCAGGACG CGAGCCGCCA GCGGCGGGAC 200

CCATCGACGA CTTCCCGGGG CGACAGGAGC AGCCCCGAGA GCCAGGGCGA 250

GCGCCCGTTC CAGGTGGCCG GACCGCCCGC CGCGTCCGCG CCGCGCTCCC 300

TGCAGGCAAC GGGAGACGCC CCCGCGCAGC GCGAGCGCCT CAGCGCGGCC 350

GCTCGCTCTC CCCATCGAGG GACAACTTT TCCCAAACCC GATCCGAGCC 400

CTTGGACCAA ACTCGCCTGC GCCGAGAGCC GTCCGCGTAG AGCGCTCCGT 450

CTCCGGCGAG	ATG	TCC	GAG	CGC	AAA	GAA	GGC	AGA	GGC	AAA	490
	Met	Ser	Glu	Arg	Lys	Glu	Gly	Arg	Gly	Lys	
	1				5					10	

GGG	AAG	GGC	AAG	AAG	AAG	GAG	CGA	GGC	TCC	GGC	AAG	AAG	529
Gly	Lys	Gly	Lys	Lys	Lys	Glu	Arg	Gly	Ser	Gly	Lys	Lys	
				15					20				

CCG	GAG	TCC	GCG	GCG	GGC	AGC	CAG	AGC	CCA	GCC	TTG	CCT	568
Pro	Glu	Ser	Ala	Ala	Gly	Ser	Gln	Ser	Pro	Ala	Leu	Pro	
	25					30						35	

CCC	CAA	TTG	AAA	GAG	ATG	AAA	AGC	CAG	GAA	TCG	GCT	GCA	607
Pro	Gln	Leu	Lys	Glu	Met	Lys	Ser	Gln	Glu	Ser	Ala	Ala	
			40					45					

GGT	TCC	AAA	CTA	GTC	CTT	CGG	TGT	GAA	ACC	AGT	TCT	GAA	646
Gly	Ser	Lys	Leu	Val	Leu	Arg	Cys	Glu	Thr	Ser	Ser	Glu	
	50					55					60		

TAC	TCC	TCT	CTC	AGA	TTC	AAG	TGG	TTC	AAG	AAT	GGG	AAT	685
Tyr	Ser	Ser	Leu	Arg	Phe	Lys	Trp	Phe	Lys	Asn	Gly	Asn	
			65				70					75	

GAA	TTG	AAT	CGA	AAA	AAC	AAA	CCA	CAA	AAT	ATC	AAG	ATA	724
Glu	Leu	Asn	Arg	Lys	Asn	Lys	Pro	Gln	Asn	Ile	Lys	Ile	
						80					85		

FIG. 3A

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CAA	AAA	AAG	CCA	GGG	AAG	TCA	GAA	CTT	CGC	ATT	AAC	AAA	763
Gln	Lys	Lys	Pro	Gly	Lys	Ser	Glu	Leu	Arg	Ile	Asn	Lys	
	90					95					100		
GCA	TCA	CTG	GCT	GAT	TCT	GGA	GAG	TAT	ATG	TGC	AAA	GTG	802
Ala	Ser	Leu	Ala	Asp	Ser	Gly	Glu	Tyr	Met	Cys	Lys	Val	
			105					110					
ATC	AGC	AAA	TTA	GGA	AAT	GAC	AGT	GCC	TCT	GCC	AAT	ATC	841
Ile	Ser	Lys	Leu	Gly	Asn	Asp	Ser	Ala	Ser	Ala	Asn	Ile	
115					120					125			
ACC	ATC	GTG	GAA	TCA	AAC	GAG	ATC	ATC	ACT	GGT	ATG	CCA	880
Thr	Ile	Val	Glu	Ser	Asn	Glu	Ile	Ile	Thr	Gly	Met	Pro	
		130					135					140	
GCC	TCA	ACT	GAA	GGA	GCA	TAT	GTG	TCT	TCA	GAG	TCT	CCC	919
Ala	Ser	Thr	Glu	Gly	Ala	Tyr	Val	Ser	Ser	Glu	Ser	Pro	
				145					150				
ATT	AGA	ATA	TCA	GTA	TCC	ACA	GAA	GGA	GCA	AAT	ACT	TCT	958
Ile	Arg	Ile	Ser	Val	Ser	Thr	Glu	Gly	Ala	Asn	Thr	Ser	
	155					160					165		
TCA	TCT	ACA	TCT	ACA	TCC	ACC	ACT	GGG	ACA	AGC	CAT	CTT	997
Ser	Ser	Thr	Ser	Thr	Ser	Thr	Thr	Gly	Thr	Ser	His	Leu	
			170					175					
GTA	AAA	TGT	GCG	GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	1036
Val	Lys	Cys	Ala	Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	
180					185					190			
GGA	GGG	GAG	TGC	TTC	ATG	GTG	AAA	GAC	CTT	TCA	AAC	CCC	1075
Gly	Gly	Glu	Cys	Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	
		195					200					205	
TCG	AGA	TAC	TTG	TGC	AAG	TGC	CCA	AAT	GAG	TTT	ACT	GGT	1114
Ser	Arg	Tyr	Leu	Cys	Lys	Cys	Pro	Asn	Glu	Phe	Thr	Gly	
			210						215				
GAT	CGC	TGC	CAA	AAC	TAC	GTA	ATG	GCC	AGC	TTC	TAC	AAG	1153
Asp	Arg	Cys	Gln	Asn	Tyr	Val	Met	Ala	Ser	Phe	Tyr	Lys	
	220					225					230		
GCG	GAG	GAG	CTG	TAC	CAG	AAG	AGA	GTG	CTG	ACC	ATA	ACC	1192
Ala	Glu	Glu	Leu	Tyr	Gln	Lys	Arg	Val	Leu	Thr	Ile	Thr	
			235					240					
GGC	ATC	TGC	ATC	GCC	CTC	CTT	GTG	GTC	GGC	ATC	ATG	TGT	1231
Gly	Ile	Cys	Ile	Ala	Leu	Leu	Val	Val	Gly	Ile	Met	Cys	
245					250					255			
GTG	GTG	GCC	TAC	TGC	AAA	ACC	AAG	AAA	CAG	CGG	AAA	AAG	1270
Val	Val	Ala	Tyr	Cys	Lys	Thr	Lys	Lys	Gln	Arg	Lys	Lys	
		260					265				270		

FIG. 3B

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CTG	CAT	GAC	CGT	CTT	CGG	CAG	AGC	CTT	CGG	TCT	GAA	CGA	1309
Leu	His	Asp	Arg	Leu	Arg	Gln	Ser	Leu	Arg	Ser	Glu	Arg	
				275						280			

AAC	AAT	ATG	ATG	AAC	ATT	GCC	AAT	GGG	CCT	CAC	CAT	CCT	1348
Asn	Asn	Met	Met	Asn	Ile	Ala	Asn	Gly	Pro	His	His	Pro	
	285					290					295		

AAC	CCA	CCC	CCC	GAG	AAT	GTC	CAG	CTG	GTG	AAT	CAA	TAC	1387
Asn	Pro	Pro	Pro	Glu	Asn	Val	Gln	Leu	Val	Asn	Gln	Tyr	
			300					305					

GTA	TCT	AAA	AAC	GTC	ATC	TCC	AGT	GAG	CAT	ATT	GTT	GAG	1426
Val	Ser	Lys	Asn	Val	Ile	Ser	Ser	Glu	His	Ile	Val	Glu	
310					315					320			

AGA	GAA	GCA	GAG	ACA	TCC	TTT	TCC	ACC	AGT	CAC	TAT	ACT	1465
Arg	Glu	Ala	Glu	Thr	Ser	Phe	Ser	Thr	Ser	His	Tyr	Thr	
		325				330						335	

TCC	ACA	GCC	CAT	CAC	TCC	ACT	ACT	GTC	ACC	CAG	ACT	CCT	1504
Ser	Thr	Ala	His	His	Ser	Thr	Thr	Val	Thr	Gln	Thr	Pro	
				340						345			

AGC	CAC	AGC	TGG	AGC	AAC	GGA	CAC	ACT	GAA	AGC	ATC	CTT	1543
Ser	His	Ser	Trp	Ser	Asn	Gly	His	Thr	Glu	Ser	Ile	Leu	
	350					355					360		

TCC	GAA	AGC	CAC	TCT	GTA	ATC	GTG	ATG	TCA	TCC	GTA	GAA	1582
Ser	Glu	Ser	His	Ser	Val	Ile	Val	Met	Ser	Ser	Val	Glu	
			365					370					

AAC	AGT	AGG	CAC	AGC	AGC	CCA	ACT	GGG	GGC	CCA	AGA	GGA	1621
Asn	Ser	Arg	His	Ser	Ser	Pro	Thr	Gly	Gly	Pro	Arg	Gly	
375					380					385			

CGT	CTT	AAT	GGC	ACA	GGA	GGC	CCT	CGT	GAA	TGT	AAC	AGC	1660
Arg	Leu	Asn	Gly	Thr	Gly	Gly	Pro	Arg	Glu	Cys	Asn	Ser	
		390					395					400	

TTC	CTC	AGG	CAT	GCC	AGA	GAA	ACC	CCT	GAT	TCC	TAC	CGA	1699
Phe	Leu	Arg	His	Ala	Arg	Glu	Thr	Pro	Asp	Ser	Tyr	Arg	
				405					410				

GAC	TCT	CCT	CAT	AGT	GAA	AGG	TAT	GTG	TCA	GCC	ATG	ACC	1738
Asp	Ser	Pro	His	Ser	Glu	Arg	Tyr	Val	Ser	Ala	Met	Thr	
	415					420					425		

ACC	CCG	GCT	CGT	ATG	TCA	CCT	GTA	GAT	TTC	CAC	ACG	CCA	1777
Thr	Pro	Ala	Arg	Met	Ser	Pro	Val	Asp	Phe	His	Thr	Pro	
			430					435					

AGC	TCC	CCC	AAA	TCG	CCC	CCT	TCG	GAA	ATG	TCT	CCA	CCC	1816
Ser	Ser	Pro	Lys	Ser	Pro	Pro	Ser	Glu	Met	Ser	Pro	Pro	
440					445					450			

FIG._3C

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GTG	TCC	AGC	ATG	ACG	GTG	TCC	AAG	CCT	TCC	ATG	GCG	GTC	1855
Val	Ser	Ser	Met	Thr	Val	Ser	Lys	Pro	Ser	Met	Ala	Val	
		455					460					465	
AGC	CCC	TTC	ATG	GAA	GAA	GAG	AGA	CCT	CTA	CTT	CTC	GTG	1894
Ser	Pro	Phe	Met	Glu	Glu	Glu	Arg	Pro	Leu	Leu	Leu	Val	
				470					475				
ACA	CCA	CCA	AGG	CTG	CGG	GAG	AAG	AAG	TTT	GAC	CAT	CAC	1933
Thr	Pro	Pro	Arg	Leu	Arg	Glu	Lys	Lys	Phe	Asp	His	His	
	480					485					490		
CCT	CAG	CAG	TTC	AGC	TCC	TTC	CAC	CAC	AAC	CCC	GCG	CAT	1972
Pro	Gln	Gln	Phe	Ser	Ser	Phe	His	His	Asn	Pro	Ala	His	
			495					500					
GAC	AGT	AAC	AGC	CTC	CCT	GCT	AGC	CCC	TTG	AGG	ATA	GTG	2011
Asp	Ser	Asn	Ser	Leu	Pro	Ala	Ser	Pro	Leu	Arg	Ile	Val	
505					510					515			
GAG	GAT	GAG	GAG	TAT	GAA	ACG	ACC	CAA	GAG	TAC	GAG	CCA	2050
Glu	Asp	Glu	Glu	Tyr	Glu	Thr	Thr	Gln	Glu	Tyr	Glu	Pro	
		520					525					530	
GCC	CAA	GAG	CCT	GTT	AAG	AAA	CTC	GCC	AAT	AGC	CGG	CGG	2089
Ala	Gln	Glu	Pro	Val	Lys	Lys	Leu	Ala	Asn	Ser	Arg	Arg	
				535					540				
GCC	AAA	AGA	ACC	AAG	CCC	AAT	GGC	CAC	ATT	GCT	AAC	AGA	2128
Ala	Lys	Arg	Thr	Lys	Pro	Asn	Gly	His	Ile	Ala	Asn	Arg	
	545					550					555		
TTG	GAA	GTG	GAC	AGC	AAC	ACA	AGC	TCC	CAG	AGC	AGT	AAC	2167
Leu	Glu	Val	Asp	Ser	Asn	Thr	Ser	Ser	Gln	Ser	Ser	Asn	
			560					565					
TCA	GAG	AGT	GAA	ACA	GAA	GAT	GAA	AGA	GTA	GGT	GAA	GAT	2206
Ser	Glu	Ser	Glu	Thr	Glu	Asp	Glu	Arg	Val	Gly	Glu	Asp	
570					575					580			
ACG	CCT	TTC	CTG	GGC	ATA	CAG	AAC	CCC	CTG	GCA	GCC	AGT	2245
Thr	Pro	Phe	Leu	Gly	Ile	Gln	Asn	Pro	Leu	Ala	Ala	Ser	
		585					590					595	
CTT	GAG	GCA	ACA	CCT	GCC	TTC	CGC	CTG	GCT	GAC	AGC	AGG	2284
Leu	Glu	Ala	Thr	Pro	Ala	Phe	Arg	Leu	Ala	Asp	Ser	Arg	
				600					605				
ACT	AAC	CCA	GCA	GGC	CGC	TTC	TCG	ACA	CAG	GAA	GAA	ATC	2323
Thr	Asn	Pro	Ala	Gly	Arg	Phe	Ser	Thr	Gln	Glu	Glu	Ile	
	610					615					620		
CAG	GCC	AGG	CTG	TCT	AGT	GTA	ATT	GCT	AAC	CAA	GAC	CCT	2362
Gln	Ala	Arg	Leu	Ser	Ser	Val	Ile	Ala	Asn	Gln	Asp	Pro	
			625					630					

FIG. 3D

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ATT GCT GTA TAAACCTA AATAACACA TAGATTCACC TGTAAACTT 2410
Ile Ala Val
635 637

TATTTTATAT AATAAGTAT TCCACCTTAA ATTAAACAAT TTATTTTATT 2460

TTAGCAGTTC TGCAAATAAA AAAAAAAAAA 2490

FIG._3E

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GCGCCTGCOCT CCAACCTGCG GGCGGGAGGT GGGTGGCTGC GGGGCAATTG 50

AAAAAGAGCC GGCGAGGAGT TCCCCGAAAC TTGTTGGAAC TCCGGGCTCG 100

CGCGGAGGCC AGGAGCTGAG CGGCGGCGGC TGCCGGACGA TGGGAGCGTG 150

AGCAGGAOOG TGATAACCTC TCCCCGATCG GGTTGCGAGG GCGCCGGGCA 200

GAGGCCAGGA CGCGAGCOGC CAGCGGCGGG ACCCATCGAC GACTTCCCGG 250

GGCGACAGGA GCAGCCCCGA GAGCCAGGGC GAGCGCCCGT TCCAGGTGGC 300

CGGACCGCCC GCCGCGTCCG CGCCGCGCTC CCTGCAGGCA ACGGGAGACG 350

CCCCCGCGCA GCGCGAGCGC CTCAGCGCGG CCGCTCGCTC TCCCCATCGA 400

GGGACAAACT TTTCCCAAAC CCGATCCGAG CCCTTGGACC AAActCGCCT 450

GCGCCGAGAG CCGTCCGCGT AGAGCGCTCC GTCTCCGGCG AG ATG 495
Met
1

TCC GAG CGC AAA GAA GGC AGA GGC AAA GGG AAG GGC AAG 534
Ser Glu Arg Lys Glu Gly Arg Gly Lys Gly Lys Gly Lys
5 10

AAG AAG GAG CGA GGC TCC GGC AAG AAG CCG GAG TCC GCG 573
Lys Lys Glu Arg Gly Ser Gly Lys Lys Pro Glu Ser Ala
15 20 25

GCG GGC AGC CAG AGC CCA GCC TTG CCT CCC CAA TTG AAA 612
Ala Gly Ser Gln Ser Pro Ala Leu Pro Pro Gln Leu Lys
30 35 40

GAG ATG AAA AGC CAG GAA TCG GCT GCA GGT TCC AAA CTA 651
Glu Met Lys Ser Gln Glu Ser Ala Ala Gly Ser Lys Leu
45 50

GTC CTT CGG TGT GAA ACC AGT TCT GAA TAC TCC TCT CTC 690
Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu
55 60 65

AGA TTC AAG TGG TTC AAG AAT GGG AAT GAA TTG AAT CGA 729
Arg Phe Lys Trp Phe Lys Asn Gly Asn Glu Leu Asn Arg
70 75

FIG. 4A

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AAA	AAC	AAA	CCA	CAA	AAT	ATC	AAG	ATA	CAA	AAA	AAG	CCA	768
Lys	Asn	Lys	Pro	Gln	Asn	Ile	Lys	Ile	Gln	Lys	Lys	Pro	
80					85					90			
GGG	AAG	TCA	GAA	CTT	CGC	ATT	AAC	AAA	GCA	TCA	CTG	GCT	807
Gly	Lys	Ser	Glu	Leu	Arg	Ile	Asn	Lys	Ala	Ser	Leu	Ala	
	95					100						105	
GAT	TCT	GGA	GAG	TAT	ATG	TGC	AAA	GTG	ATC	AGC	AAA	TTA	846
Asp	Ser	Gly	Glu	Tyr	Met	Cys	Lys	Val	Ile	Ser	Lys	Leu	
			110						115				
GGA	AAT	GAC	AGT	GCC	TCT	GCC	AAT	ATC	ACC	ATC	GTG	GAA	885
Gly	Asn	Asp	Ser	Ala	Ser	Ala	Asn	Ile	Thr	Ile	Val	Glu	
	120					125					130		
TCA	AAC	GAG	ATC	ATC	ACT	GGT	ATG	CCA	GCC	TCA	ACT	GAA	924
Ser	Asn	Glu	Ile	Ile	Thr	Gly	Met	Pro	Ala	Ser	Thr	Glu	
			135					140					
GGA	GCA	TAT	GTG	TCT	TCA	GAG	TCT	CCC	ATT	AGA	ATA	TCA	963
Gly	Ala	Tyr	Val	Ser	Ser	Glu	Ser	Pro	Ile	Arg	Ile	Ser	
145					150					155			
GTA	TCC	ACA	GAA	GGA	GCA	AAT	ACT	TCT	TCA	TCT	ACA	TCT	1002
Val	Ser	Thr	Glu	Gly	Ala	Asn	Thr	Ser	Ser	Ser	Thr	Ser	
		160					165					170	
ACA	TCC	ACC	ACT	GGG	ACA	AGC	CAT	CTT	GTA	AAA	TGT	GCG	1041
Thr	Ser	Thr	Thr	Gly	Thr	Ser	His	Leu	Val	Lys	Cys	Ala	
				175					180				
GAG	AAG	GAG	AAA	ACT	TTC	TGT	GTG	AAT	GGA	GGG	GAG	TGC	1080
Glu	Lys	Glu	Lys	Thr	Phe	Cys	Val	Asn	Gly	Gly	Glu	Cys	
	185					190					195		
TTC	ATG	GTG	AAA	GAC	CTT	TCA	AAC	CCC	TCG	AGA	TAC	TTG	1119
Phe	Met	Val	Lys	Asp	Leu	Ser	Asn	Pro	Ser	Arg	Tyr	Leu	
			200					205					
TGC	AAG	TGC	CCA	AAT	GAG	TTT	ACT	GGT	GAT	CGC	TGC	CAA	1158
Cys	Lys	Cys	Pro	Asn	Glu	Phe	Thr	Gly	Asp	Arg	Cys	Gln	
210					215					220			
AAC	TAC	GTA	ATG	GCC	AGC	TTC	TAC	AGT	ACG	TCC	ACT	CCC	1197
Asn	Tyr	Val	Met	Ala	Ser	Phe	Tyr	Ser	Thr	Ser	Thr	Pro	
		225					230					235	
TTT	CTG	TCT	CTG	CCT	GAA	TAGGA	GCATGCTCAG	TTGGTGCTGC	1240				
Phe	Leu	Ser	Leu	Pro	Glu								
				240	241								
TTTCTTGTTG	CTGCATCTCC	CCTCAGATTC	CACCTAGAGC	TAGATGTGTC	1290								

FIG. 4B

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TTACCAGATC TAATATTGAC TGCCTCTGCC TGTCGCATGA GAACATTAAC 1340
AAAAGCAATT GTATTACTTC CTCTGTTTCG CACTAGTTGG CTCTGAGATA 1390
CTAATAGGTG TGTGAGGCTC CGGATGTTTC TGGAATTGAT ATTGAATGAT 1440
GTGATACAAA TTGATAGTCA ATATCAAGCA GTGAAATATG ATAATAAAGG 1490
CATTTCAAAG TCTCACTTTT ATTGATAAAA TAAAATCAT TCTACTGAAC 1540
AGTCCATCTT CTTTATACAA TGACCACATC CTGAAAAGGG TGTGCTAAG 1590
CTGTAACCGA TATGCACTTG AAATGATGGT AAGTTAATTT TGATTCAGAA 1640
TGTGTTATTT GTCACAAATA AACATAATAA AAGGAGTTCA GATGTTTTTC 1690
TTCATTAACC AAAAAAAAAA AAAAA 1715

FIG. 4C

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GAGGCGCCTG CCTCCAACCT GCGGGCGGGA GGTGGGTGGC TCGGGGGCAA 50
 TTGAAAAGA GCGGGCGAGG AGTTCCCGA AACTTGTTGG AACTCCGGGC 100
 TCGCGCGGAG GCCAGGAGCT GAGCGGCGGC GGCTGCCGGA CGATGGGAGC 150
 GTGAGCAGGA CGGTGATAAC CTCTCCCGA TCGGGTTGCG AGGGCGCCGG 200
 GCAGAGGCCA GGACGCGAGC CGCCAGCGGC GGGACCCATC GACGACTTCC 250
 CGGGGCGACA GGAGCAGCCC CGAGAGCCAG GGCGAGCGCC CGTTCCAGGT 300
 GGCCGGACCG CCCGCCGCGT CCGCGCCGCG CTCCCTGCAG GCAACGGGAG 350
 ACGCCCCCGC GCAGCGCGAG CGCCTCAGCG CGGCCGCTCG CTCTCCCAT 400
 CGAGGGACAA ACTTTTCCCA AACCCGATCC GAGCCCTTGG ACCAAACTCG 450
 CCTGCGCCGA GAGCCGTCCG CGTAGAGCGC TCCGTCTCCG GCGAG AT 497
 Met
 1
 G TCC GAG CGC AAA GAA GGC AGA GGC AAA GGG AAG GGC AAG 537
 Ser Glu Arg Lys Glu Gly Arg Gly Lys Gly Lys Gly Lys
 5 10
 AAG AAG GAG CGA GGC TCC GGC AAG AAG CCG GAG TCC GCG 576
 Lys Lys Glu Arg Gly Ser Gly Lys Lys Pro Glu Ser Ala
 15 20 25
 GCG GGC AGC CAG AGC CCA GCC TTG CCT CCC CAA TTG AAA 615
 Ala Gly Ser Gln Ser Pro Ala Leu Pro Pro Gln Leu Lys
 30 35 40
 GAG ATG AAA AGC CAG GAA TCG GCT GCA GGT TCC AAA CTA 654
 Glu Met Lys Ser Gln Glu Ser Ala Ala Gly Ser Lys Leu
 45 50
 GTC CTT CGG TGT GAA ACC AGT TCT GAA TAC TCC TCT CTC 693
 Val Leu Arg Cys Glu Thr Ser Ser Glu Tyr Ser Ser Leu
 55 60 65
 AGA TTC AAG TGG TTC AAG AAT GGG AAT GAA TTG AAT CGA 732
 Arg Phe Lys Trp Phe Lys Asn Gly Asn Glu Leu Asn Arg
 70 75

FIG. 5A

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AAA AAC AAA CCA CAA AAT ATC AAG ATA CAA AAA AAG CCA 771	
Lys Asn Lys Pro Gln Asn Ile Lys Ile Gln Lys Lys Pro	
80	85 90
GGG AAG TCA GAA CTT CGC ATT AAC AAA GCA TCA CTG GCT 810	
Gly Lys Ser Glu Leu Arg Ile Asn Lys Ala Ser Leu Ala	
95	100 105
GAT TCT GGA GAG TAT ATG TGC AAA GTG ATC AGC AAA TTA 849	
Asp Ser Gly Glu Tyr Met Cys Lys Val Ile Ser Lys Leu	
110	115
GGA AAT GAC AGT GCC TCT GCC AAT ATC ACC ATC GTG GAA 888	
Gly Asn Asp Ser Ala Ser Ala Asn Ile Thr Ile Val Glu	
120	125 130
TCA AAC GAG ATC ATC ACT GGT ATG CCA GCC TCA ACT GAA 927	
Ser Asn Glu Ile Ile Thr Gly Met Pro Ala Ser Thr Glu	
135	140
GGA GCA TAT GTG TCT TCA GAG TCT CCC ATT AGA ATA TCA 966	
Gly Ala Tyr Val Ser Ser Glu Ser Pro Ile Arg Ile Ser	
145	150 155
GTA TCC ACA GAA GGA GCA AAT ACT TCT TCA TCT ACA TCT 1005	
Val Ser Thr Glu Gly Ala Asn Thr Ser Ser Ser Thr Ser	
160	165 170
ACA TCC ACC ACT GGG ACA AGC CAT CTT GTA AAA TGT GCG 1044	
Thr Ser Thr Thr Gly Thr Ser His Leu Val Lys Cys Ala	
175	180
GAG AAG GAG AAA ACT TTC TGT GTG AAT GGA GGG GAG TGC 1083	
Glu Lys Glu Lys Thr Phe Cys Val Asn Gly Gly Glu Cys	
185	190 195
TTC ATG GTG AAA GAC CTT TCA AAC CCC TCG AGA TAC TTG 1122	
Phe Met Val Lys Asp Leu Ser Asn Pro Ser Arg Tyr Leu	
200	205
TGC AAG TGC CCA AAT GAG TTT ACT GGT GAT CGC TGC CAA 1161	
Cys Lys Cys Pro Asn Glu Phe Thr Gly Asp Arg Cys Gln	
210	215 220
AAC TAC GTA ATG GCC AGC TTC TAC AAG GCG GAG GAG CTG 1200	
Asn Tyr Val Met Ala Ser Phe Tyr Lys Ala Glu Glu Leu	
225	230 235
TAC CAG AAG AGA GTG CTG ACC ATA ACC GGC ATC TGC ATC 1239	
Tyr Gln Lys Arg Val Leu Thr Ile Thr Gly Ile Cys Ile	
240	245
GCC CTC CTT GTG GTC GGC ATC ATG TGT GTG GTG GCC TAC 1278	
Ala Leu Leu Val Val Gly Ile Met Cys Val Val Ala Tyr	
250	255 260

FIG. 5B

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TGC	AAA	ACC	AAG	AAA	CAG	CGG	AAA	AAG	CTG	CAT	GAC	CGT	1317
Cys	Lys	Thr	Lys	Lys	Gln	Arg	Lys	Lys	Leu	His	Asp	Arg	
			265					270					
CTT	CGG	CAG	AGC	CTT	CGG	TCT	GAA	CGA	AAC	AAT	ATG	ATG	1356
Leu	Arg	Gln	Ser	Leu	Arg	Ser	Glu	Arg	Asn	Asn	Met	Met	
275					280					285			
AAC	ATT	GCC	AAT	GGG	CCT	CAC	CAT	CCT	AAC	CCA	CCC	CCC	1395
Asn	Ile	Ala	Asn	Gly	Pro	His	His	Pro	Asn	Pro	Pro	Pro	
		290					295					300	
GAG	AAT	GTC	CAG	CTG	GTG	AAT	CAA	TAC	GTA	TCT	AAA	AAC	1434
Glu	Asn	Val	Gln	Leu	Val	Asn	Gln	Tyr	Val	Ser	Lys	Asn	
			305						310				
GTC	ATC	TCC	AGT	GAG	CAT	ATT	GTT	GAG	AGA	GAA	GCA	GAG	1473
Val	Ile	Ser	Ser	Glu	His	Ile	Val	Glu	Arg	Glu	Ala	Glu	
	315					320					325		
ACA	TCC	TTT	TCC	ACC	AGT	CAC	TAT	ACT	TCC	ACA	GCC	CAT	1512
Thr	Ser	Phe	Ser	Thr	Ser	His	Tyr	Thr	Ser	Thr	Ala	His	
			330					335					
CAC	TCC	ACT	ACT	GTC	ACC	CAG	ACT	CCT	AGC	CAC	AGC	TGG	1551
His	Ser	Thr	Thr	Val	Thr	Gln	Thr	Pro	Ser	His	Ser	Trp	
340					345					350			
AGC	AAC	GGA	CAC	ACT	GAA	AGC	ATC	CTT	TCC	GAA	AGC	CAC	1590
Ser	Asn	Gly	His	Thr	Glu	Ser	Ile	Leu	Ser	Glu	Ser	His	
		355					360					365	
TCT	GTA	ATC	GTG	ATG	TCA	TCC	GTA	GAA	AAC	AGT	AGG	CAC	1629
Ser	Val	Ile	Val	Met	Ser	Ser	Val	Glu	Asn	Ser	Arg	His	
			370						375				
AGC	AGC	CCA	ACT	GGG	GGC	CCA	AGA	GGA	CGT	CTT	AAT	GGC	1668
Ser	Ser	Pro	Thr	Gly	Gly	Pro	Arg	Gly	Arg	Leu	Asn	Gly	
	380					385					390		
ACA	GGA	GGC	CCT	CGT	GAA	TGT	AAC	AGC	TTC	CTC	AGG	CAT	1707
Thr	Gly	Gly	Pro	Arg	Glu	Cys	Asn	Ser	Phe	Leu	Arg	His	
			395					400					
GCC	AGA	GAA	ACC	CCT	GAT	TCC	TAC	CGA	GAC	TGT	CCT	CAT	1746
Ala	Arg	Glu	Thr	Pro	Asp	Ser	Tyr	Arg	Asp	Ser	Pro	His	
405					410					415			
AGT	GAA	AGG	TAAAA	CCGAAGGCAA	AGCTACTGCA	GAGGAGAAAC	1790						
Ser	Glu	Arg											
		420											

FIG. 5C

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TCAGTCAGAG AATCCCTGTG AGCACCTGCG GTCTCACCTC AGGAAATCTA 1840
CTCTAATCAG AATAAGGGGC GGCAGTTACC TGTCTAGGA GTGCTCCTAG 1890
TTGATGAAGT CATCTCTTTG TTTGACGGAA CTTATTTCTT CTGAGCTTCT 1940
CTCGTCGTCC CAGTGACTGA CAGGCAACAG ACTCTTAAAG AGCTGGGATG 1990
CTTTGATGCG GAAGGTGCAG CACATGGAGT TTCCAGCTCT GGCCATGGGC 2040
TCAGACCCAC TCGGGGTCTC AGTGTCTCA GTTGTAACAT TAGAGAGATG 2090
GCATCAATGC TTGATAAGGA CCCTTCTATA ATTCCAATTG CCAGTTATCC 2140
AAACTCTGAT TCGGTGGTCG AGCTGGCCTC GTGTTCTTAT CTGCTAACCC 2190
TGTCTTACCT TCCAGCCTCA GTTAAGTCAA ATCAAGGGCT ATGTCATTGC 2240
TGAATGTCAT GGGGGGCAAC TGCTTGCCCT CCACCCTATA GTATCTATTT 2290
TATGAAATTC CAAGAAGGGA TGAATAAATA AATCTCTTGG ATGCTGCGTC 2340
TGGCAGTCTT CACGGGTGGT TTTCAAAGCA GAAAAAAAAA AAAAAAAAAA 2390
AAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA A 2431

FIG. 5D

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16	1	M	S	E	R	K	E	G	R	G	K	G	K	K	E	R	G	S	G	K	K	P	E	S	A	A	G	S	Q	S	P	A	L	P	P	R	L	K	E	M	K	S	Q	E	S	A	A	G			
11	1	M	S	E	R	K	E	G	R	G	K	G	K	K	E	R	G	S	G	K	K	P	E	S	A	A	G	S	Q	S	P	A	L	P	P	Q	L	K	E	M	K	S	Q	E	S	A	A	G			
76	1	M	S	E	R	K	E	G	R	G	K	G	K	K	E	R	G	S	G	K	K	P	E	S	A	A	G	S	Q	S	P	A	L	P	P	Q	L	K	E	M	K	S	Q	E	S	A	A	G			
84	1	M	S	E	R	K	E	G	R	G	K	G	K	K	E	R	G	S	G	K	K	P	E	S	A	A	G	S	Q	S	P	A	L	P	P	Q	L	K	E	M	K	S	Q	E	S	A	A	G			
78	1	M	S	E	R	K	E	G	R	G	K	G	K	K	E	R	G	S	G	K	K	P	E	S	A	A	G	S	Q	S	P	A	L	P	P	Q	L	K	E	M	K	S	Q	E	S	A	A	G			
16	51	S	K	L	V	L	R	C	E	T	S	S	E	Y	S	S	L	R	F	K	W	F	K	N	G	N	E	L	N	R	K	N	K	P	Q	N	I	K	I	Q	K	K	P	G	K	S	E	L	R	I	N
11	51	S	K	L	V	L	R	C	E	T	S	S	E	Y	S	S	L	R	F	K	W	F	K	N	G	N	E	L	N	R	K	N	K	P	Q	N	I	K	I	Q	K	K	P	G	K	S	E	L	R	I	N
76	51	S	K	L	V	L	R	C	E	T	S	S	E	Y	S	S	L	R	F	K	W	F	K	N	G	N	E	L	N	R	K	N	K	P	Q	N	I	K	I	Q	K	K	P	G	K	S	E	L	R	I	N
84	51	S	K	L	V	L	R	C	E	T	S	S	E	Y	S	S	L	R	F	K	W	F	K	N	G	N	E	L	N	R	K	N	K	P	Q	N	I	K	I	Q	K	K	P	G	K	S	E	L	R	I	N
78	51	S	K	L	V	L	R	C	E	T	S	S	E	Y	S	S	L	R	F	K	W	F	K	N	G	N	E	L	N	R	K	N	K	P	Q	N	I	K	I	Q	K	K	P	G	K	S	E	L	R	I	N
16	101	K	A	S	L	A	D	S	G	E	Y	M	C	K	V	I	S	K	L	G	N	D	S	A	S	A	N	I	T	I	V	E	S	N	E	I	I	T	Q	M	P	A	S	T	E	G	A	Y	V	S	
11	101	K	A	S	L	A	D	S	G	E	Y	M	C	K	V	I	S	K	L	G	N	D	S	A	S	A	N	I	T	I	V	E	S	N	E	I	I	T	Q	M	P	A	S	T	E	G	A	Y	V	S	
76	101	K	A	S	L	A	D	S	G	E	Y	M	C	K	V	I	S	K	L	G	N	D	S	A	S	A	N	I	T	I	V	E	S	N	E	I	I	T	Q	M	P	A	S	T	E	G	A	Y	V	S	
84	101	K	A	S	L	A	D	S	G	E	Y	M	C	K	V	I	S	K	L	G	N	D	S	A	S	A	N	I	T	I	V	E	S	N	E	I	I	T	Q	M	P	A	S	T	E	G	A	Y	V	S	
78	101	K	A	S	L	A	D	S	G	E	Y	M	C	K	V	I	S	K	L	G	N	D	S	A	S	A	N	I	T	I	V	E	S	N	E	I	I	T	Q	M	P	A	S	T	E	G	A	Y	V	S	
16	151	E	S	P	I	R	I	S	V	S	T	E	G	A	N	T	S	S	S	T	S	T	S	T	T	G	T	S	H	L	V	K	C	A	E	K	E	K	T	F	C	V	N	G	G	E	C	F	M	V	K
11	151	E	S	P	I	R	I	S	V	S	T	E	G	A	N	T	S	S	S	T	S	T	S	T	T	G	T	S	H	L	V	K	C	A	E	K	E	K	T	F	C	V	N	G	G	E	C	F	M	V	K
76	151	E	S	P	I	R	I	S	V	S	T	E	G	A	N	T	S	S	S	T	S	T	S	T	T	G	T	S	H	L	V	K	C	A	E	K	E	K	T	F	C	V	N	G	G	E	C	F	M	V	K
84	151	E	S	P	I	R	I	S	V	S	T	E	G	A	N	T	S	S	S	T	S	T	S	T	T	G	T	S	H	L	V	K	C	A	E	K	E	K	T	F	C	V	N	G	G	E	C	F	M	V	K
78	151	E	S	P	I	R	I	S	V	S	T	E	G	A	N	T	S	S	S	T	S	T	S	T	T	G	T	S	H	L	V	K	C	A	E	K	E	K	T	F	C	V	N	G	G	E	C	F	M	V	K

FIG.-6A

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16	201	DLSNP	SRYLCKCQ	QPGFTGA	RCTEN	VP	MKVQNQ	-----	EK	AEELYQKR	VLT
11	201	DLSNP	SRYLCKCQ	PNFTGDR	CQNYVMA	SFYK	HLG	IEFME		AEELYQKR	VLT
76	201	DLSNP	SRYLCKCQ	PNFTGDR	CQNYVMA	SFYK	-----	-----		AEELYQKR	VLT
84	201	DLSNP	SRYLCKCQ	PNFTGDR	CQNYVMA	SFYK	-----	-----		AEELYQKR	VLT
78	201	DLSNP	SRYLCKCQ	PNFTGDR	CQNYVMA	SFYK	-----	-----		AEELYQKR	VLT
16	248	ITGIC	IALLVVGI	MCVVAYC	KTKKQR	KKLHD	RLRQ	SLRSE	RNN	MMN	IANG
11	251	ITGIC	IALLVVGI	MCVVAYC	KTKKQR	KKLHD	RLRQ	SLRSE	RNN	MMN	IANG
76	243	ITGIC	IALLVVGI	MCVVAYC	KTKKQR	KKLHD	RLRQ	SLRSE	RNN	MMN	IANG
84	243	ITGIC	IALLVVGI	MCVVAYC	KTKKQR	KKLHD	RLRQ	SLRSE	RNN	MMN	IANG
16	298	PHHPN	PPPEN	VQLVNQ	YVSKNV	ISS	EHIVER	EAE	TSF	STSHY	TSTAHHST
11	301	PHHPN	PPPEN	VQLVNQ	YVSKNV	ISS	EHIVER	EAE	TSF	STSHY	TSTAHHST
76	293	PHHPN	PPPEN	VQLVNQ	YVSKNV	ISS	EHIVER	EAE	TSF	STSHY	TSTAHHST
84	293	PHHPN	PPPEN	VQLVNQ	YVSKNV	ISS	EHIVER	EAE	TSF	STSHY	TSTAHHST
16	348	TVTQT	PSHWS	NGHTES	ILSE	SHSV	IVM	SSVEN	SRH	SSPTGG	PRQLNGT
11	351	TVTQT	PSHWS	NGHTES	ILSE	SHSV	IVM	SSVEN	SRH	SSPTGG	PRQLNGT
76	343	TVTQT	PSHWS	NGHTES	ILSE	SHSV	IVM	SSVEN	SRH	SSPTGG	PRQLNGT
84	343	TVTQT	PSHWS	NGHTES	ILSE	SHSV	IVM	SSVEN	SRH	SSPTGG	PRQLNGT

FIG.-6B

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16	398	GGPRECN	SFLRHARE	TPDSYRD	SPHSERY	VSAMTT	PARMS	PVDFHT	PSSP	
11	401	GGPRECN	SFLRHARE	TPDSYRD	SPHSERY	VSAMTT	PARMS	PVDFHT	PSSP	
76	393	GGPRECN	SFLRHARE	TPDSYRD	SPHSERY	VSAMTT	PARMS	PVDFHT	PSSP	
84	393	GGPRECN	SFLRHARE	TPDSYRD	SPHSER				
16	446	KSPPPSE	MSPPVSS	MTVSM	PSMAV	SPFM	EEER	PLLL	VTPPRLREKKFDHHP	
11	451	KSPPPSE	M8PPVSS	MTVSM	PSMAV	SPFM	EEER	PLLL	VTPPRLREKKFDHHP	
76	443	KSPPPSE	M8PPVSS	MTVSK	PSMAV	SPFM	EEER	PLLL	VTPPRLREKKFDHHP	
16	498	QFSSF	HHPAH	DSNSL	PASPL	RIVED	EEYET	TQEY	EPAQEPVKKLANSR	
11	501	QFSSF	HHPAH	DSNSL	PASPL	RIVED	EEYET	TQEY	EPAQEPVKKLANSR	
76	493	QFSSF	HHPAH	DSNSL	PASPL	RIVED	EEYET	TQEY	EPAQEPVKKLANSR	
16	548	RAKRT	KPNQ	GHIAN	RLE	VD	SNTSS	QSSNSE	SETE	DERVGEDTPFLGIQNPL
11	551	RAKRT	KPNQ	GHIAN	RLE	VD	SNTSS	QSSNSE	SETE	DERVGEDTPFLGIQNPL
76	543	RAKRT	KPNQ	GHIAN	RLE	VD	SNTSS	QSSNSE	SETE	DERVGEDTPFLGIQNPL
16	598	AASLE	ATPA	FRLA	DSRT	NPAG	RFST	QEEI	Q.....	
11	601	AASLE	ATPA	FRLA	DSRT	NPAG	RFST	QEEI	QARLSSVIANQDP	
76	593	AASLE	ATPA	FRLA	DSRT	NPAG	RFST	QEEI	QARLSSVIANQDP	

FIG._6C

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1 GGGTACCATGGGTTCGGTGGAGCGCGTTTCCCGCCTGAGCGCAACTAGCGGC
51 GGGTCGTGGGCACCTCCAGAAAAGATCCCGCACCATCCTCCAGGATCCAA
101 TGGCCTTGGAGAGAGGGCTGCAGGGCCACGGACATTGCTGACTCTTCAG
151 AACGTGCTGACATGGAGCCAGGTAGACTGAAATTATCATGTGTCCAAATT
201 AAAATTGCATACTTCAAGGATTATTTGAAGGACTATTCTTAGACCCTTTT
251 AAGAAGATTTAAAGAAAAACCACTCGGCCCTGAGTGCGGCGAGGACCCTG

301 TTTGTGGATGTGGAGGAGCGCGGGCCGGAGGCCATGGACGTGAAGGAGAG
1 M D V K E R

351 GAAGCCTTACCGCTCGCTGACCCGGCGCCGCGACGCCGAGCGCCGCTACA
7 K P Y R S L T R R R D A E R R Y T

401 CCAGCTCGTCCGCGGACAGCGAGGAGGGCAAAGCCCCGCAGAAATCGTAC
24 S S S A D S E E G K A P Q K S Y

451 AGCTCCAGCGAGACCCTGAAGGCCTACGACCAGGACGCCCGCCTAGCCTA
40 S S S E T L K A Y D Q D A R L A Y

501 TGGCAGCCGCGTCAAGGACATTGTGCCGCGAGGAGGCCGAGGAATTCTGCC
57 G S R V K D I V P Q E A E E F C R

551 GCACAGGTGCCAACTTCACCCTGCGGGAGCTGGGGCTGGAAGAAGTAACG
74 T G A N F T L R E L G L E E V T

601 CCCCCTCACGGGACCCTGTACCGGACAGACATTGGCCTCCCCCACTGCGG
90 P P H G T L Y R T D I G L P H C G

651 CTA CTCCATGGGGGCTGGCTCTGATGCCGACATGGAGGCTGACACGGTGC
107 Y S M G A G S D A D M E A D T V L

701 TGTCCCCTGAGCACCCCGTGCGTCTGTGGGGCCGGAGCACACGGTCAGGG
124 S P E H P V R L W G R S T R S G

751 CGCAGCTCCTGCCTGTCCAGCCGGGCCAATTCCAATCTCACACTCACCGA
140 R S S C L S S R A N S N L T L T D

801 CACCGAGCATGAAAACACTGAGACTGATCATCCGGGCGGCCTGCAGAACC
157 T E H E N T E T D H P G G L Q N H

851 ACGCGCGGCTCCGGACGCCGCGCCGCGCGCTCTCGCACGCCCACACCCCC
174 A R L R T P P P P L S H A H T P

901 AACCAGCACCACGCGGCCTCCATTA ACTCCCTGAACCGGGGCAACTTCAC
190 N Q H H A A S I N S L N R G N F T

951 GCCGAGGAGCAACCCCGAGCCCGGCCCCACGGACCACTCGCTCTCCGGAG
207 P R S N P S P A P T D H S L S G E

1001 AGCCCCCTGCCGGCGGCGCCCAGGAGCCTGCCACGCCCAGGAGAACTGG
224 P P A G G A Q E P A H A Q E N W

1051 CTGCTCAACAGCAACATCCCCCTGGAGACCAGAAACCTAGGCAAGCAGCC
240 L L N S N I P L E T R N L G K Q P

FIG. 7A

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1101 ATTCCTAGGGACATTGCAGGACAACCTCATTGAGATGGACATTCTCGGCG
257 F L G T L Q D N L I E M D I L G A

1151 CCTCCCGCCATGATGGGGCTTACAGTGACGGGCACTTCCTCTTCAAGCCT
274 S R H D G A Y S D G H F L F K P

1201 GGAGGCACCTCCCCGCTCTTCTGCACCACATCACCAGGGTACCCACTGAC
290 G G T S P L F C T T S P G Y P L T

1251 GTCCAGCACAGTGTACTCTCCTCCGCCCCGACCCCTGCCCCGCAGCACCT
307 S S T V Y S P P P R P L P R S T F

1301 TCGCCCGGGCCGGCCTTTAACCTCAAGAAGCCCTCCAAGTACTGTAAGTGG
324 A R P A F N L K K P S K Y C N W

1351 AAGTGCGCAGCCCTGAGCGCCATCGTCATCTCAGCCACTCTGGTCATCCT
340 K C A A L S A I V I S A T L V I L

1401 GCTGGCATACTTTGTGGCCATGCACCTGTTTGGCCTAAACTGGCACCTGC
357 L A Y F V A M H L F G L N W H L Q

1451 AGCCGATGGAGGGGCGAGATGTATGAGATCACGGAGGACACAGCCAGCAGT
374 P M E G Q M Y E I T E D T A S S

1501 TGGCCTGTGCCAACCGACGTCTCCCTATACCCCTCAGGGGGCACTGGCTT
390 W P V P T D V S L Y P S G G T G L

1551 AGAGACCCCTGACAGGAAAGGCAAAGGAACACAGAAGGAAAGCCCAGTA
407 E T P D R K G K G T T E G K P S S

1601 GTTTCTTTCCAGAGGACAGTTTCATAGATTCTGGAGAAATTGATGTGGGA
424 F F P E D S F I D S G E I D V G

1651 AGGCGAGCTTCCCAGAAGATTCTCCTGGCACTTTCTGGAGATCTCAAGT
440 R R A S Q K I P P G T F W R S Q V

1701 GTTCATAGACCATCCTGTGCATCTGAAATTCAATGTGTCTCTGGGAAAGG
457 F I D H P V H L K F N V S L G K A

1751 CAGCCCTGGTTGGCATTATGGCAGAAAAGGCCTCCCTCCTTCACATACA
474 A L V G I Y G R K G L P P S H T

1801 CAGTTTGA CTTTGTGGAGCTGCTGGATGGCAGGAGGCTCCTAACCCAGGA
490 Q F D F V E L L D G R R L L T Q E

1851 GGCGCGGAGCCTAGAGGGGACCCCGCGCCAGTCTCGGGGAACTGTGCCCC
507 A R S L E G T P R Q S R G T V P P

1901 CCTCCAGCCATGAGACAGGCTTCATCCAGTATTTGGATTTCAGGAATCTGG
524 S S H E T G F I Q Y L D S G I W

1951 CACTTGGCTTTTTTACAATGACGGAAAGGAGTCAGAAGTGGTTTCCTTTCT
540 H L A F Y N D G K E S E V V S F L

FIG. 7B

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2001 CACCACTGCCATTGCCTTGCCTCCCCGATTGAAAGAGATGAAAAGCCAGG
557 T T A I A L P P R L K E M K S Q E

2051 AATCGGCTGCAGGTTCCAACTAGTCCTTCGGTGTGAAACCAGTTCTGAA
574 S A A G S K L V L R C E T S S E

2101 TACTCCTCTCTCAGATTCAAGTGGTTCAAGAATGGGAATGAATTGAATCG
590 Y S S L R F K W F K N G N E L N R

2151 AAAAAACAAACCACAAAATATCAAGATACAAAAAAGCCAGGGAAGTCAG
607 K N K P Q N I K I Q K K P G K S E

2201 AACTTCGCATTAAACAAAGCATCACTGGCTGATTCTGGAGAGTATATGTGC
624 L R I N K A S L A D S G E Y M C

2251 AAAGTGATCAGCAAATTAGGAAATGACAGTGCCTCTGCCAATATCACCAT
640 K V I S K L G N D S A S A N I T I

2301 CGTGGAATCAAACGAGATCATCACTGGTATGCCAGCCTCAACTGAAGGAG
657 V E S N E I I T G M P A S T E G A

2351 CATATGTGTCTTCAGAGTCTCCCATTAGAATATCAGTATCCACAGAAGGA
674 Y V S S E S P I R I S V S T E G

2401 GCAAATACTTCTTCATCTACATCTACATCCACCACTGGGACAAGCCATCT
690 A N T S S S T S T S T T G T S H L

2451 TGTAATAATGTGCGGAGAAGGAGAAAACCTTTCTGTGTGAATGGAGGGGAGT
707 V K (C) A E K E K T F (C) V N G G E (C)

2501 GCTTCATGGTGAAAGACCTTTCAAACCCCTCGAGATACTTGTGCAAGTGC
724 F M V K D L S N P S R Y L (C) K (C)

2551 CCAAATGAGTTTACTGGTGATCGCTGCCAAACTACGTAATGGCCAGCTT
740 P N E F T G D R (C) Q N Y V M A S F

2601 CTACAGTACGTCCACTCCCTTTCTGTCTCTGCCTGAATAGGAGCATGCTC
757 Y S T S T P F L S L P E

2651 AGTTGGTGCTGCTTTCTTGTTGCTGCATCTCCCCTCAGATTCCACCTAGA

2701 GCTAGATGTGTCTTACCAGATCTAATATTGACTGCCTCTGCCTGTGCGCAT
2751 GAGAACATTAAACAAAAGCAATTGTATTACTTCCTCTGTTCGCGACTAGTT
2801 GGCTCTGAGATACTAATAGGTGTGTGAGGCTCCGGATGTTTCTGGAATTG
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3051 TTTGATTCAAGATGTGTTATTTGTACAAATAAACATAATAAAAGGAAAA
3101 AAAAAAAAAA

FIG. 7C

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SUBSTITUTE SHEET (RULE 26)

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29 / 29

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 158 Q G D K A V V S F E P S A A P T P K N R I F A F S
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 208 Q P Q T T E T N L Q T A P K L S T S T S T T G T S
 1201 GCCATCTTGTAATAATGTGGGAGAGGAGAAACTTTCTGTGTGAATGGAGGGGAGTGCTTCATGGTGAAAGACC
 233 H L V K C A E K E K T F C V N G G E C F H V K D L
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 283 S F Y S T S T P F L S L P E O
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 1726 TCTTCTTTATACAAAGACCATCTCTGAAAGGGTGTGCTAAGCTGTAAACCGATATGCACCTTGAAATGATGGTA
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EGF
-like

FIG. 8B

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	Glu	Arg	Gly	Ser	Gly	Lys	Lys	Pro	Glu	Ser	Ala	Ala	Gly	Ser	Gln
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	Leu Asn Gly Thr Gly Gly Pro Arg Glu Cys Asn Ser Phe Leu Arg		
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